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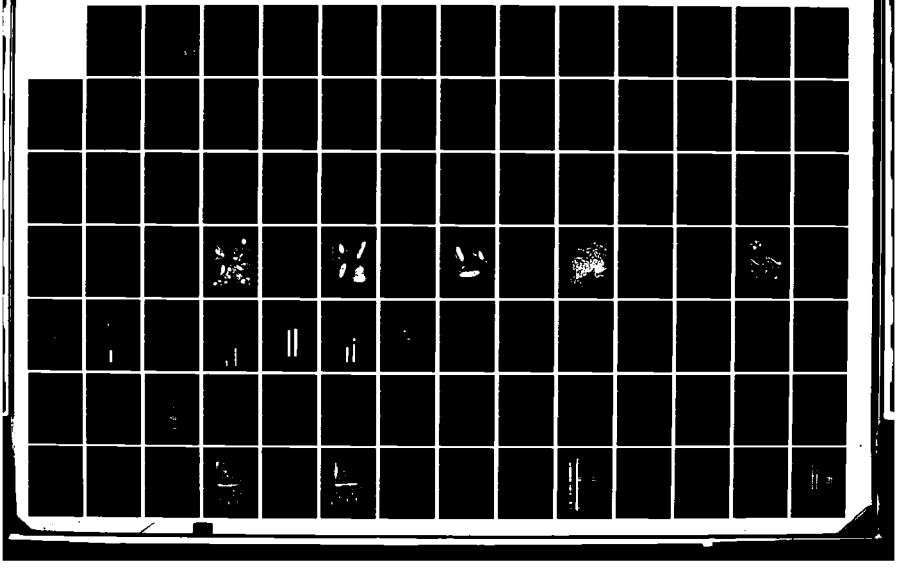

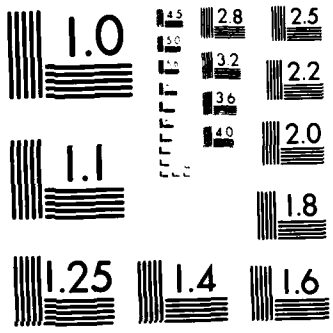

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STUDIES ON TYPHUS AND SPOTTED FEVER

ANNUAL PROGRESS REPORT

by

Charles L. Wisseman, Jr., M.D.

February, 1980
(For the period 1 July 1978 to 30 June 1979)

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(1) Two major arms of protective typhus immunity have been investigated: (a) cell mediated immunity which restricts intracellular rickettsial growth local at site of lesions and (b) antibody-macrophage clearance of extracellular rickettsiae. In a R. mooseri-mouse model, T-lymphocytes, but not B-lymphocytes, non-specifically activated macrophages or immune serum, restricted rickettsial replication in the spleen. In electron microscope studies with human blood monocyte-derived macrophages in culture, virulent R. prowazekii destroyed the phagosomal membrane without lysosomal fusion, escaped into the cytoplasm and...

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multiplied freely. When pretreated with human immune serum, R. prowazekii was retained in the phagosome, lysosomal fusion was rapid and the organisms quickly underwent degeneration. Soluble factors in the supernatant fluids from immunologically specific or mitogen stimulated human blood leukocytes had two separable effects on R. prowazekii-infected human somatic (fibroblast, endothelial), but not chick, mouse or monkey, cells in culture: (a) intracellular antirickettsial action and (b) cytotoxic action on infected cells. (2) By selective extraction under non-denaturing conditions followed by immunoprecipitation, two protein components of R. prowazekii could be isolated. (3) By DNA hybridization and PAGE protein profiles, R. canada was found to occupy a separate status within the genus Rickettsia equivalent to the typhus and spotted fever groups. By radio-isotope protein labeling and radiofluorography of PAGE protein profiles, the Gilliam strain, Kato strain and Karp-like Pakistan isolate of R. tsutsugamushi were found to have unique patterns, as well as some common protein components. Two-dimensional protein separation (iso-electric focussing followed by polyacrylamide gel electrophoresis) detected apparent differences between the virulent Breinl and attenuated E strains of R. prowazekii. Cholesteral did not appear to be the receptor on CE cells for R. prowazekii uptake.

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I. IMMUNOLOGICAL STUDIES

Through both *in vitro* and *in vivo* studies, we seem to be developing substantial evidence for two major arms of the immune response to typhus infection (and probably some other rickettsial infections): (1) cell-mediated immune mechanisms which can control rickettsial proliferation intracellularly at the local sites of infection in tissues and (2) an antibody-macrophage mechanism which, if it is operationally significant *in vivo*, is probably more likely to be effective as a clearance mechanism (as from blood), since immune serum alone does not control rickettsial replication in local tissue sites. It remains to be determined if one, or the other, or both are required of effective vaccines.

The following report deals with progress along the following lines.

(1) *In vitro* studies of intracellular *antirickettsial* action and *cytotoxic* action on infected cells by supernatant fluids from stimulated human blood leukocytes.

(2) Electron microscopic studies of mechanisms by which *R. prowazekii* circumvents the microbicidal action of human monocyte-derived macrophages *in vitro* and the events which lead to the intravacuolar destruction of immune serum opsonized rickettsiae.

(3) *In vivo* studies in a *R. mooseri*-mouse model which demonstrates the failure of macrophages activated by classical CMI mechanisms to control rickettsial proliferation in the absence of antibodies.

(4) *In vivo* studies in a *R. mooseri*-mouse model which demonstrates that immune T-lymphocytes, but not immune serum, restrict rickettsial proliferation locally in the spleen.

A. *Mediators of Cell-Mediated Immunity Effective Against Intracellular Rickettsiae: In Vitro Studies of Intracellular Antirickettsial and Cytotoxic Actions on Infected Human Cells by Supernatant Fluids from Stimulated Human Blood Leukocytes.* With the growing evidence that a substantial portion of immunity in typhus is mediated through T-lymphocytes by mechanisms *other* than the classical CMI of tuberculosis and *Listeria* which depends on the activation of macrophages by lymphocyte products, we searched for other mechanisms with apparent success. Preliminary observations recorded in the previous annual report suggested that human leukocytes from persons who had experienced typhus infection, when stimulated *in vitro* by killed *R. prowazekii* antigen, elaborated factor(s) into the supernatant fluid that appeared to exert unmistakable effects on rickettsia-infected human, but not chicken embryo, fibroblastic cells *in vitro*. This suggested that there might be a mechanism by which intracellular rickettsial growth can be controlled in the tissues at the sites of infection, as in endothelial cells which appear to be the major target cells for the obligate intracellular parasitic bacteria of the genus *Rickettsia*. This would constitute a unique immunological mechanism of both general and specific importance. Accordingly, considerable

effort has been expended in the past year to establish and define the phenomenon. Improved assay procedures have been devised which draw heavily upon the methods and knowledge from our earlier studies on basic growth characteristics of rickettsiae and methods of quantitation. The studies defining the phenomenon per se are nearing completion and are presented in detail below. They suggest at this time a final common pathway phenomenon in which production of the mediator may be triggered either by immunologically specific or by non-specific mitogen signals and also suggest that both of the types of action recognized, i.e., antirickettsial and cytotoxic, are expressed through changes produced in the host cell. The specific nature of the mediators and the cells which produce them and their precise mode of action are subjects for future studies.

METHODS

1. Rickettsial Strains. The rickettsial species and strains employed in this study are recorded in Table 1. With the exception of the Flying Squirrel strain of R. prowazekii and the R. tsutsugamushi strains, the strains had been plaque purified in these laboratories (Wisseman, C.L. Jr., A.D. Waddell and M.R. Jones, unpublished method) and were prepared for use as 20% yolk sac suspensions in brain heart infusion broth and distributed in small convenient volumes to borosilicate glass ampoules, which were flame sealed, frozen rapidly in a dry ice-ethanol mixture and stored at -70°C. Some preparations were highly purified by a batch sucrose - albumin:immunoprecipitation - gradient (usually renograffin) procedure in long routine use in these laboratories (Wisseman, C.L. Jr., and A.D. Waddell, unpublished method). Routine titration was by plaque count in CE cell monolayers after the method of Wike et al. (13). In some stances, the RLB/PFU ratio, to estimate the proportion of viable organisms, was performed by methods developed in these laboratories (Wisseman, C.L. Jr., A.D. Waddell and M.L. Cremer, unpublished method).

2. Production of human leukocyte supernatant fluids. All procedures were carried out aseptically and without the use of antibiotics. Blood (50 to 500 ml) was collected into heparin (10 units per ml). To this, 1 ml 6% dextran (T-250) in saline was added for each 10 ml of blood. After incubation of the container at a 45° angle at 37°C for approximately 40 minutes, the leukoplasma was collected. The leukocytes were sedimented at 4°C by centrifugation at 200 xg for 10 min, washed 3x in RPMI medium by repeated resuspension and centrifugation and resuspended in RPMI medium containing 10% human AB plasma (inactivated at 56°C for 1 h). The resuspended cells were counted in a hemocytometer and adjusted to a concentration of 10^6 mononuclear cells per ml. Either formalin-killed, purified Rickettsia prowazekii antigen (ET) or phytohemagglutinin (PHA) was added to a final concentration of 1 µg/ml. For control supernatant (CS) no antigen or mitogen was added. (In early studies, the cell suspension was divided into 3 parts: (1) one portion for control supernatant; (2) one portion with added R. prowazekii antigen; and (3) one portion with added PHA. Later, for the preparation of large quantities of supernatants, a small portion was reserved for control and the remainder stimulated with either R. prowazekii antigen or PHA.) The cell suspensions were added in 1 ml volumes to Falcon tissue culture tubes (#3033) and incubated at 37°C in a 5% CO_2: 95% air atmosphere for 18-24 h. After incubation, the

Table 1 Source, passage history, and other pertinent information on the strains employed in this study

Organism/strain	Source	Passage history[a]	Plaque Purified	Ref
Rickettsia prowazekii				
Breinl	Human	E155/TC3/E3	Yes	44
Madrid E	Human	CRD-3[b]/TC3/E4	Yes	11
Bur X-16	Human	TC5/E2	Yes	c
GV-F12[d]	Flying squirrel	E7	No	4
Rickettsia mooseri (*R. typhi*)				
Wilmington	Rat	E42/TC3/E3	Yes	28
Ethio AZ-306	Rat	TC5/E3	Yes	c
Rickettsia canada	Tick	E16/TC3/E1	Yes	e
Rickettsia rickettsii				
Sheila Smith	Human	GP2/E6/TC3/E2/TC4/E6	Yes	f
Ripley	Human	GP1/CE1/E1	Yes	g
Rickettsia tsutsugamushi				
Gilliam		E141/M2/E2	No	h
JC-472 (karp-like)		M3/E10	No	i
Karp		E107	No	j

a Abbreviations: E, Yolk sac passage; TC, tissue culture plaque purification passage; GP, guinea pig passage; BA, blood agar passage.

b Experimental vaccine lot CRD-3 (C.L. Wisseman, Jr., unpublished data).

c C.L. Wisseman, *et al.*, unpublished data.

d Furnished by F.M. Bozeman, Bureau of Biologics, Food and Drug Administration, Bethesda, Md.

e ATCC stock contributed by Dr. Richard Ormsbee.

f Furnished by F.M. Bozeman, Bureau of Biologics at passage level 2GP/3y.s. 9/28/62.

g Isolated in these laboratories

h Obtained from Walter Reed, Dept. of Ricket. Diseases. Jan '62 - yolk sac 132P.

i Isolated from *Tatera*, in Sialkot, Pakistan 10/3/64.

j Obtained from Walter Reed, Dept. of Ricket. Diseases. Jan '62 - yolk sac 98P.

like suspensions were pooled and the cells were removed by centrifugation at 200 g for 10 min at 4°C. The clear supernatant fluids were filtered through a 0.22 µm Millipore filter and were distributed in convenient quantities in rubber stoppered vaccine vials, frozen in dry ice-alcohol mixture and stored at -70°C.

At this stage of the investigation, which was directed primarily at confirming the phenomena and defining some of their general characteristics, no detailed systematic studies were performed on the production of active supernatants. It was established in one experiment that removal of the bulk of the cells which would attach to plastic tissue culture dishes (adherent cells) did not reduce the activity of the supernatant fluid subsequently obtained by stimulation of the lymphocyte enriched suspension. In other experiments, it was found that the activity of the supernatants was as great after incubation of leukocytes for only 18-24 h at 37°C as it was after 5 days of incubation, at a time when cells were capable of actively incorporating ^{3}H-thymidine in a conventional lymphoblast transformation assay. Hence, we adopted the practice of incubating for 18-24 h. A few early attempts at producing active supernatant fluids by stimulating the leukocytes in larger volumes in different kinds of vessels were unsuccessful. Hence, for the time being, we continued to incubate them in small volumes in round bottomed plastic tissue culture tubes. These, and other variables, will obviously require detailed systematic study in the future. However, because all of the work, including the tests for activity, are tedious, slow and labor-intensive and the resources were limited, we chose first to establish the validity and reproducibility of the phenomena and to define some of their general characteristics with supernatants produced by a method which had proven to work before expending large amounts of time and effort in attempts at refinement.

All of the studies performed here were with leukocytes derived from the blood of human subjects who were either non-immune to typhus or other known rickettsial disease by history and serology or who were immune to typhus by virtue of prior infection. Some of the latter had complex histories of exposure to other rickettsial antigens, both live and killed. However, the bulk of the studies reported here, unless otherwise specified, were performed with leukocyte supernatants from subjects who experienced only typhus fever caused by Rickettsia prowazekii. For convenience, the following abbreviations will be used to designate supernatant fluids used in the studies reported here:

CM: Control tissue culture medium not exposed to leukocytes. This was D/2 TC medium + 10% FCS described below.

CS: RPMI medium + 10% human AB plasma which had been incubated with unstimulated leukocytes, either from non-immune or from typhus-immune subjects, under the same conditions as for the stimulated leukocytes but without added antigen or mitogen.

ET-S: RPMI medium + 10% human AB plasma in which leukocytes from a typhus-immune subject were incubated in the presence of killed R. prowazekii (Breinl) antigen. (Immunologically specific stimulation)

PHA-S: RPMI medium + 10% human AB plasma in which leukocytes from a non-immune subject had been incubated with PHA. (Immunologically non-specific stimulation)

For use in tests, CS, ET-S and PHA-S, all containing RPMI medium + 10% human AB plasma, were diluted 1:2 with our standard half-strength Dulbecco's medium containing 0.1% glucose and 10% FCS.

3. Tests for antirickettsial activity of leukocyte supernatant fluids. Preliminary tests for leukocyte supernatant activity were performed in a modification of our basic slide chamber culture method used previously to study the infection cycle of rickettsiae in cells and the action of antibiotics on the growth of these organisms (14, 15, 16, 18). In this type of test supernatant fluids were added to cultures after the cells had been infected with rickettsiae. This sequence is referred to as post-infection treatment or simply post-treatment.

Host cells were grown in plastic flasks in our basic medium which consists of half-strength Dulbecco's medium with Earle's salts containing 0.1% glucose and 10% fetal calf serum (FCS). This same medium was used as a diluent for rickettsiae and for leukocyte supernatants. No antibiotics were used in these studies.

Trypsinized host cells infected in suspension (Wisseman, C.L. Jr., and A.D. Waddell, to be published) were distributed to Lab-Tek culture chamber slides (about 6×10^4 cells per chamber on 4-chamber slides) and were allowed to attach for about 6 h at 32°C. After attachment, the medium was removed and replaced with CM, CS, ET-S and PHA-S, as indicated in a given experiment. Incubation was continued at 32°C in an atmosphere of 5% CO_2: 95% air. At intervals from 0 to 120 h (usually to 48 or 72 h), slides were removed, fixed and stained as previously described (15). For all organisms except R. tsutsugamushi, the stain was the Giménez stain. For R. tsutsugamushi, Giemsa stain was employed. The slide cultures were examined microscopically for gross evidence of cell loss, which was scored as 0, ca. 25%, ca. 50%, ca. 75% or ca. 100% as compared with the corresponding uninfected control without leukocyte supernatant. The rickettsiae in 300 cells were counted. The data were recorded as follows:

p_i: the percent cells infected.
N: the average number of rickettsiae per cell.
N_i: the average number of rickettsiae per infected cell.

These data, plotted graphically either as standard growth curves (15, 16, 18) or transformed in various ways, provided much information about the effects of leukocyte supernatants on intracellular rickettsiae.

When it became clear that there were two distinct kinds of effects of stimulated supernatants on infected cells, i.e., (1) an intracellular antirickettsial action and (2) a cytotoxic effect, two assay systems were devised which measured each effect separately. These are described below. The events leading up to their development are described somewhat more fully in Results.

a. Intracellular Antirickettsial Assay. This assay, as the one described above, depended upon the microscopic enumeration of rickettsiae in host cells, but differs in that the host cells were treated with leukocyte supernatant or control media prior to infection with rickettsiae and is designated as the pre-treatment test. Subconfluent cultures of cells in flasks were incubated at 32°C in a 5% CO_2: 95% air atmosphere with CM, CS, ET-S and/or PHA-S diluted 1:2 in D/2 + 10% FCS (CM). After incubation, the fluids were removed. The monolayers were washed 3x with CM. The cells were removed in Trypsin/EDTA and sedimented by centrifugation at 200 g for 5 min. The pellets were resuspended in CM; the viable cell count (trypan blue exclusion) was determined. For uninfected controls, 0.6 ml aliquots of cells at a concentration of 1×10^5 per ml were distributed to each chamber of 4-chamber Lab-Tek slides. For infected cells, the cells were infected in suspension for 30 min at 32°C (Wisseman, C.L. Jr., and A.D. Waddell, to be published), washed twice in CM by centrifugation, finally resuspended in CM to a concentration of 1×10^5 viable cells per ml and distributed to slide chambers as described above. After incubation for 6 h at 32°C in a 5% CO_2: 95% air atmosphere, the medium was replaced with fresh CM and incubation was continued under the same conditions. Slides were removed at intervals (usually 0, 16, 24, 40 and 48 h), fixed, stained and counted as described above.

b. Cytotoxicity assay. The cytotoxicity assay finally adopted was one patterned after the microcytotoxicity assay developed by Lewis et al. (6) for the action of lymphotoxins. It depends upon the direct microscopic counting of cells remaining in microtiter wells after incubation with control or stimulated leukocyte supernatants. In the usual case in which cells were first infected and then exposed to supernatants, i.e., post-treatment, cells were trypsinized from flasks, infected in suspension and distributed to each well of 96-well, flat bottomed tissue culture microtiter plates, 0.1 ml per well of suspension containing 4×10^3/ml of cells which were either uninfected or infected in suspension as above. After 6 h incubation at 32°C in 5% CO_2: 95% air, the medium was removed from each well and replaced with CM, CS, ET-S or PHA-S. Incubation was continued. At intervals (usually 0, 16, 24, 40 and 48 h) after the addition of the leukocyte supernatants, trays were processed for examination by fixing the remaining cells in methanol for 10 minutes, staining with 0.1% crystal violet for 10 minutes, washing and drying. The number of cells per well was determined by counting under a binocular dissecting microscope.

In some instances, cytotoxicity was sought for in cells which had been treated with supernatants before infection, i.e., pre-treatment. In this case, cells were pre-treated and infected as described in "a" above for antirickettsial activity and then distributed to well as just described. The incubation medium, however, was CM, i.e., regular D/2 + 10% FCS tissue culture medium.

RESULTS

1. A complex action of stimulated human leukocyte supernatant fluids on rickettsia-infected cells in vitro: possible host cell specific intracellular antirickettsial and cytotoxic actions. WI-38 diploid human cells and secondary chicken embryo fibroblasts were infected in suspension with R. prowazekii (Breinl) and distributed to slide culture chambers in our

conventional rickettsial growth system (Wisseman, C.L. Jr., and A.D. Waddell, in preparation; 15). Six hours later, when the cells had attached, the medium was replaced with (1) the supernatant fluid from human tuphus immune leukocytes which had been stimulated with killed R. prowazekii antigen (ET-S) diluted with an equal quantity of TC medium, (2) the supernatant fluid from unstimulated human typhus immune leukocytes (CS) diluted with an equal quantity of TC medium or (3) TC medium (CM) alone. Once added, the leukocyte products were continuously present during the remainder of the experiment. The slide chamber cultures were incubated at 32°C in a humid atmosphere of 5% CO_2: 95% air. At intervals, usually up to 48-72 h, slides were removed, fixed, stained and counted.

The growth cycle of R. prowazekii in WI-38 cells incubated in the presence of supernatant fluid from unstimulated typhus immune human leukocytes was indistinguishable from that in the presence of TC medium alone which in turn followed the pattern previously described for R. prowazekii (15, 16). In marked contrast, cultures incubated in the presence of supernatant fluids from typhus-immune human leukocytes which had been stimulated with killed R. prowazekii antigen (ET-S) showed a dramatic effect on both rickettsiae and host cells. After a lag period of some hours, usually between about 12 and 24 h, the percent of cells with detectable rickettsiae (p_i) began a progressive, sharp decline to reach only a fraction of the initial infection rate. A break occurred at the same time in the growth curve when plotted as $\log_2$ average number of rickettsiae per cell ($\log_2 N$). The effect was much less pronounced when growth curves were plotted as $\log_2$ average number of rickettsiae per infected cell ($\log_2 N_i$). At the same time, a progressive loss of cells from the ET-S treated infected cultures was noted, following a temporal pattern similar to the loss of rickettsiae. On the other hand, when R. prowazekii-infected chicken embryo fibroblasts were cultured in the presence of the same ET-S, there was no demonstrable effect on either rickettsiae or host cells. These observations were reproducible when experiments were repeated with the same ET-S preparation or with ET-S preparations from the stimulated leukocytes from different persons who had experienced previous typhus infections. Figure 1, the results of a single experiment, is representative of these experiments. Results similar to those obtained with irradiated WI-38 cells were obtained with either irradiated or unirradiated F-1000 human foreskin fibroblasts (see below). In a single experiment, mononuclear cells from a typhus-immune subject, which were depleted of plastic adherent cells, produced, on stimulation with killed R. prowazekii antigen, supernatants which displayed identical actions.

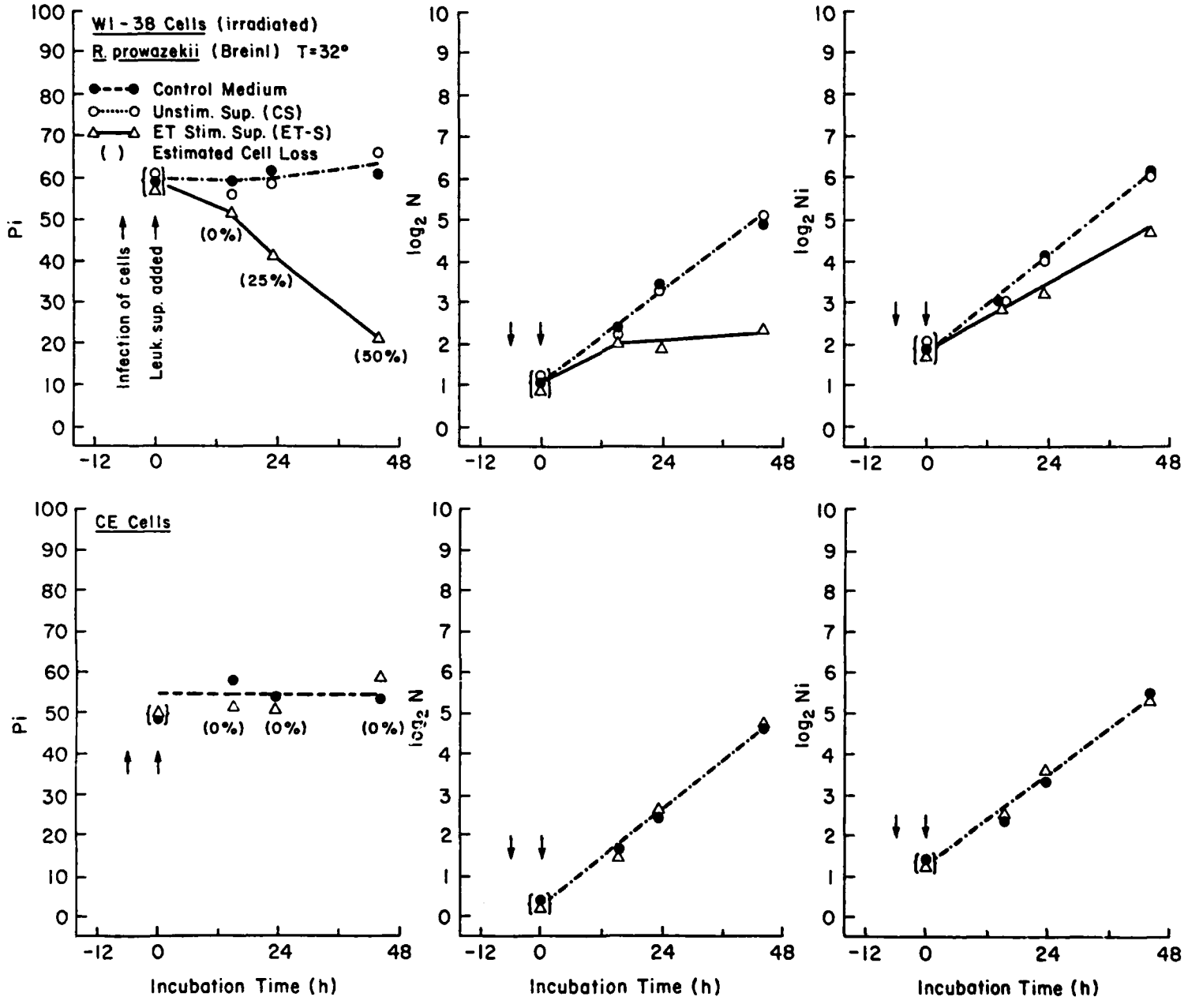
Thus, the supernatant fluids from typhus-immune human leukocytes (possibly lymphocytes) which had been stimulated with killed R. prowazekii antigen had a complex set of effects when added to R. prowazekii-infected human, but not chicken embryo, fibroblasts. These effects appeared after a lag phase of several hours, were progressive at least through 48 h and appeared to consist of (1) an antirickettsial action on intracellular rickettsiae and (2) a cytotoxic action on infected cultures. In a minority of infected and treated cells, however, rickettsial growth appeared to be relatively uninhibited. Because similar results could have been obtained by selective loss of infected cells as rickettsial growth proceeded over a period of a few hours, it was not possible to prove beyond doubt in this system that two separate actions were occurring. New, more specific, assay systems were required (see below).

FIGURE 1

Apparent Host-Specific Antirickettsial and Cytotoxic Actions on R. prowazekii (Breinl)-Infected Cells (Irradiated) Treated 6 h Post-Infection with Supernatant Fluid from R. prowazekii-Stimulated Typhus-Immune Human Leukocyte.

Note that the growth of R. prowazekii, in terms of per cent cells infected (p_i), average number of rickettsiae per cell (N) and average number of rickettsiae per infected cell (N_i), followed expected, established patterns in chicken embryo fibroblasts (CE cells) and human diploid fibroblastic cells (WI-38 cells) in the presence of control medium and the supernatant fluids from unstimulated typhus-immune human leukocytes. However, in WI-38 cells in the presence of R. prowazekii stimulated typhus-immune leukocyte supernatant (ET-S), the per cent cells infected (p_i) began a progressive decline after a lag of a few hours, accompanied by visible evidence of cell loss. After a similar lag, the growth of rickettsiae expressed as N ceased (and in some experiments the curve actually declined). When only infected cells (N_i) were counted, no such break was evident, although in this experiment the slope was less than the control. In other experiments, the slope of $\log_2 N_i$ vs time more closely approximated that of the controls. No such effect on growth was apparent in CE cells.

FIGURE 1


WI-38 Cells (irradiated)
R. prowazekii (Breinl) T=32°
Control Medium
Unstim. Sup. (CS)
ET Stim. Sup. (ET-S)
( ) Estimated Cell Loss
Infection of cells
Leuk. sup. added
(0%)
(25%)
(50%)
Pi
log2 N
log2 Ni
CE Cells
(0%)
(0%)
(0%)
Incubation Time (h)

Identical results to those described above for supernatants from typhus-immune human leukocytes stimulated with killed R. prowazekii antigen were obtained with supernatants from non-immune human leukocytes stimulated with PHA (PHA-S) (see Figure 2). Thus, human leukocytes, when stimulated either by an immunologically specific mechanism or by a non-specific T-cell mitogen like PHA, produce soluble factors (? lymphokines) which display similar antirickettsial-cytotoxic actions on human fibroblasts (host-cell specificity), which are "somatic" cells as opposed to the activated macrophages of classical cell-mediated immunity.

Pre-treatment of F-1000 cells for about 18 h with either specific (ET-S) or non-specific (PHA-S) supernatant fluids prior to infection with R. prowazekii and subsequent incubation in the absence of mononuclear cell supernatants resulted in a pronounced prompt and progressive antirickettsial action without (1) an apparent lag period or (2) apparent cytotoxic action. Figure 2 presents results obtained with PHA-S and is representative for both PHA-S and ET-S. These findings suggest (1) that the antirickettsial action is a separate process from the cytotoxic action, (2) that the antirickettsial action is expressed through an action on the host cell and (3) that the cytotoxic action required the continuous presence of active supernatants. Moreover, they suggest the feasibility of developing assay methods which are specific for either the putative antirickettsial action or cytotoxic actions.

2. Direct action of leukocyte supernatant fluids on extracellular R. prowazekii. To test supernatants for direct antirickettsial action, dilute yolk-sac suspensions of R. prowazekii (Breinl) containing 200-300 PFU/0.2 ml after mixing with an equal volume of leukocyte supernatant (control medium, control unstimulated, immune-R. prowazekii stimulated, non-immune-PHA stimulated supernatants) were incubated at 32°C for 180 minutes. Samples, removed at 0, 60, 120 and 180 minutes, were plated on CE cell monolayers for plaque titration. The CE cells had expressed neither anti-rickettsial nor cytotoxic action when infected cells were incubated in the presence of active leukocyte supernatants (see above) and, hence, were considered to be suitable for the assay of viability of rickettsiae in the presence of active supernatants. The plaque assay involves initial infection of a host cell, intracellular growth, spread to other cells in the proximity and repetition of the cycle and, thus, should be capable of detecting an inhibitory action at any point in the infection cycle. The results of two separate experiments are recorded in Table 2. In neither experiment was there any evidence of direct antirickettsial action on free organisms in suspension over a 3 h period of incubation, nor was there any subsequent interference with the infection cycle leading to plaque formation.

A second type of experiment for detection of direct antirickettsial action was performed with F-1000 human fibroblasts in slide chamber cultures. The F-1000 cells are capable of expressing both intracellular antirickettsial and cytotoxic action under appropriate conditions. Details of the experiment are presented in Table 3. The results clearly show that pre-treatment for 1 h of free R. prowazekii, freed of host cell components by prior purification, with a leukocyte supernatant capable of inducing both antirickettsial and

FIGURE 2

Antirickettsial Action in Unirradiated F-1000 Human Foreskin Fibroblast Cells Treated Before and After R. prowazekii (Breinl) Infection with Supernatant from PHA-Stimulated Non-Immune Human Leukocytes. (Compare with Fig. 1).

Note that the supernatant from PHA-stimulated typhus-non-immune leukocytes (PHA-S) caused the same pattern of antirickettsial action in R. prowazekii in unirradiated F-1000 human fibroblasts as ET-S did in irradiated WI-38 cells. Although cell loss was not scored in this particular experiment, other similar experiments with PHA-S showed the same kind of cell loss as recorded in Figure 1 with ET-S and infected WI-38 cells was observed. Pre-treatment of cells for 18 h with PHA-S prior to infection resulted in substantial antirickettsial loss without the lag period seen when supernatants was added after infection. In this case, post-infection incubation was in normal tissue culture medium. Similar antirickettsial action without lag or visible cell loss was seen in F-1000 cells treated with ET-S prior to infection.

FIGURE 2

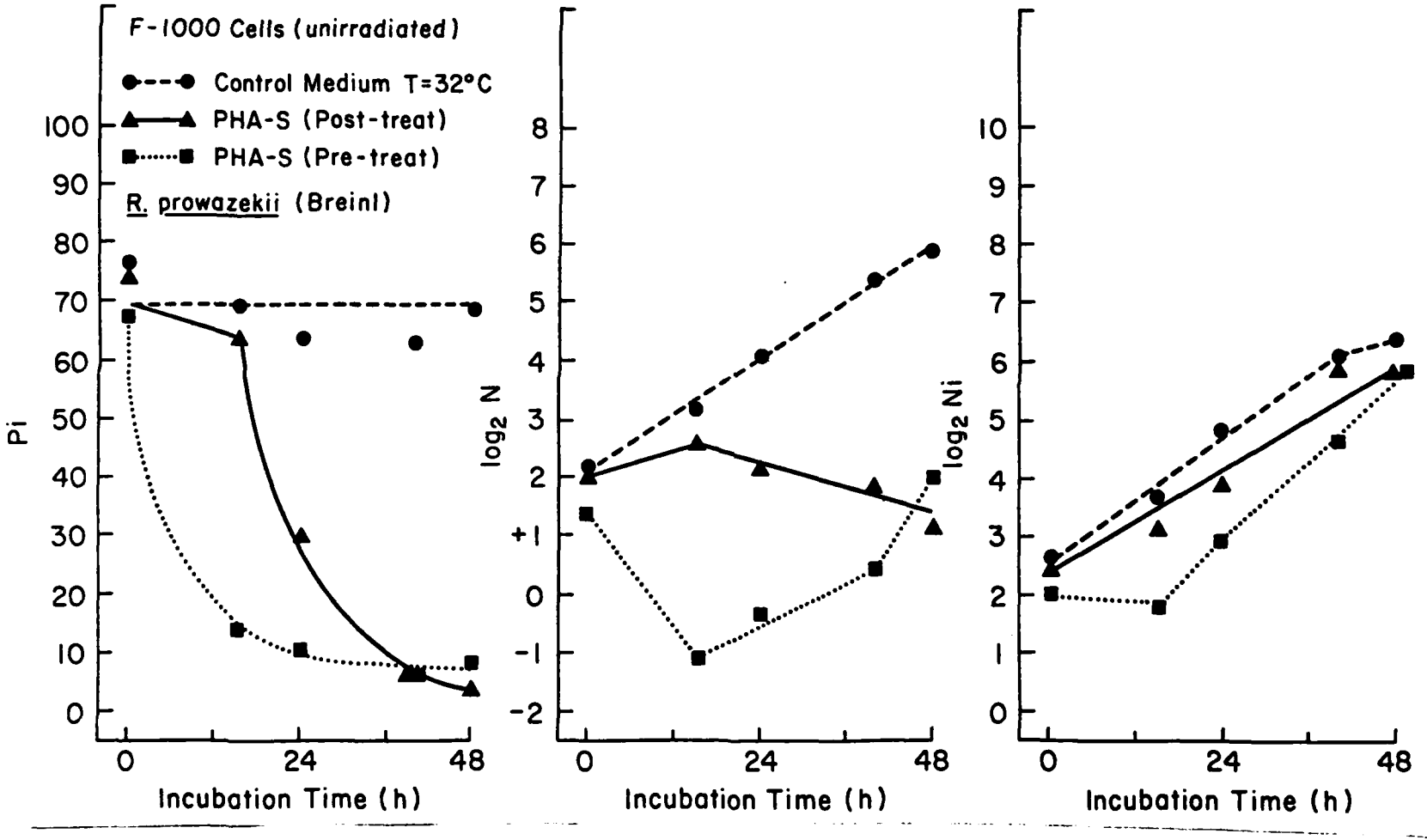

F-1000 Cells (unirradiated)
Control Medium T=32°C
PHA-S (Post-treat)
PHA-S (Pre-treat)
R. prowazekii (Breinl)
Pi
100
90
80
70
60
50
40
30
20
10
0
0
24
48
Incubation Time (h)
log2 N
8
7
6
5
4
3
+1
0
-1
-2
0
24
48
Incubation Time (h)
log2 Ni
10
9
8
7
6
5
4
3
2
1
0
0
24
48
Incubation Time (h)

Table 2 Failure of Pre-treatment of Cell-free R. prowazekii with Leukocyte Supernatant Fluids to Reduce Capacity to Form Plaques in Chicken Embryo Cell Monolayers

Incubation Time (min)[1]	Exp. No.	% Control Plaques[2] after Treatment with		
		C-S[3]	ET-S[4]	PHA-S[5]
0	Exp. 1	102	99	100
	Exp. 2	97	78	84
60	Exp. 1	101	98	104
	Exp. 2	97	82	82
120	Exp. 1	96	98	100
	Exp. 2	100	83	81
180	Exp. 1	95	98	101
	Exp. 2	100	93	95

1 Temp. = 32°C

2 Number of plaques in tissue culture medium

3 C-S = Control supernatant from unstimulated leukocytes

Exp. 1 donor (PV) non-immune
Exp. 2 donor (MBD) R. prowazekii convalescent

4 ET-S = Supernatant from leukocytes of R. prowazekii convalescent donor (MBD) stimulated with R. prowazekii antigen

5 PHA-S = Supernatant from leukocytes of typhus non-immune donor (PV) stimulated with phytohemagglutinin

Table 3 Uptake and Growth in F-1000 Human Fibroblasts of R. prowazekii (Breinl) Pre-treated in Cell-free State with Immune Leukocyte Supernatant Fluid

Incubation Time (h)	Prelim. Wash of Rickettsiae	Rickettsiae in Cells			
		p_i[2]		N[3]	
		CM	ET-S	CM	ET-S
0	0	67	71	2.0	2.0
	+	58	65	1.4	1.9
48	0	66	67	43.7	47.3
	+	69	65	47.1	46.0

1 Uptake of rickettsiae and subsequent intracellular growth were measured by slide chamber method (15 , 18). Purified R. prowazekii (Breinl) were first incubated at room temperature for 60 min with control medium (CM) or leukocyte supernatant from typhus convalescent donor (CLW) (ET-S) stimulated with R. prowazekii antigen. Half of each mixture was centrifuged and the pellet was resuspended in control medium. All 4 mixtures were used to infect F-1000 cells in slide chambers for 60 min at 32°C. After replacing fluids with control medium, slides were incubated at 32°C in 5% CO_2-air. Replicates were fixed and stained at 0 and 48 h after infection

2 p_i = % cells infected

3 N = average number of rickettsiae per cell

cytotoxic action on appropriately treated F-1000 cells, has no detectable inhibitory effect on the capacity of the rickettsiae to infect and grow within the F-1000 cells.

These experiments clearly demonstrate that active leukocyte supernatants, when incubated with extra-cellular R. prowazekii (Breinl) for 1-3 h, do not exert any direct antirickettsial action which can be detected in tests which measure uptake (infection of host cells), intracellular growth or infection cycle as measured by plaque formation. These findings, along with the apparent host cell specificity suggest that the action of leukocyte supernatants on rickettsiae may be mediated through the host cell, rather than through a direct action on the rickettsiae.

3. Development of assay methods for measurement of antirickettsial and cytotoxic actions of leukocyte supernatants. The experiments described above indicate that active leukocyte supernatants have no measurable direct action on extracellular rickettsiae but probably have two separate actions on R. prowazekii-infected diploid human cells: (a) an intracellular antirickettsial action and (b) a cytotoxic action on infected cells. It was possible to devise assay methods which specifically measured each of these actions independently.

The use of X-irradiated cells was abandoned because there was substantial, progressive loss of uninfected cells in the systems which were designed for quantitation of cell number. The two assay systems devised are described below. Specific results are given in subsequent sections, as appropriate.

a. Antirickettsial activity. It was found that F-1000 cells pretreated in flasks with supernatants for about 18 hrs, followed by washing, trypsinization, infection in suspension and distribution to Lab-Tek chamber slides permitted the detection of intracellular antirickettsial action uncomplicated by the progressive loss of cells due to the cytotoxic action when the growth kinetics were measured over a period of 48-72 h. The growth stimulatory action (see below) of some supernatants did complicate the quantitative aspects somewhat, but this usually was minimized by limiting the observations to 48 h.

b. Cytotoxic action. It was possible to measure directly the previously suspected specific cytotoxic action of active leukocyte supernatants on infected F-1000 cells.

In preliminary studies to develop an assay system for the detection and measurement of leukocyte supernatant mediated cytotoxicity, various methods which depended upon the release of radiolabeled amino acids or nucleotides or which depended upon the loss of neutral red binding capacity were tested and were found to be unsatisfactory for one reason or another. Finally, an adaptation of the original method which depends on the direct counting of crystal violet-stained residual cells in microtiter plates (6) was found to be sensitive, reproducible and reliable. Hence, all studies on cytotoxicity reported here were performed with this test.

We originally employed x-irradiated F-1000 human fibroblasts in this test to prevent distortions which might be introduced by host cell replication. However, uninfected x-irradiated cells in medium alone (CM) showed a progressive and substantial loss in numbers with respect to time. Although specific cytotoxicity could be demonstrated with x-irradiated cells, the substantial variable of cell loss due to x-irradiation caused some inconveniences and possibly some distortions for which it was difficult to compensate in all instances. When it was found that, within certain time limits and conditions, cytotoxicity measurements could be made without undue or uncorrectible replication of F-1000 human fibroblasts, a reasonable satisfactory, reproducible and reliable assay for cytotoxicity was devised which used unirradiated F-1000 human fibroblasts.

The method consisted simply of introducing an estimated 200-300 cells (uninfected or infected, as desired) into each well of the 96-well, flat-bottomed microtiter plates, allowing them to attach for 6 h, replacing the medium with the desired dilution of leukocyte supernatant in medium and incubating at 32°C in a 5% CO_2: 95% air atmosphere. Plates were removed at intervals, usually up to 48-72 h, and the cells were stained with crystal violet. The number of cells remaining attached to the bottom of each microtiter well was counted under a dissecting microscope. This method permitted the detection of growth stimulatory, cytostatic and cytotoxic responses.

These two methods, though tedious and time-consuming, permitted clearcut, separate detection and measurement of the antirickettsial and cytotoxic actions of leukocyte supernatants, as illustrated in subsequent sections.

4. Action of leukocyte supernatant fluids on uninfected host cells. The action of leukocyte supernatant fluids, whether derived from leukocytes of typhus immune subjects stimulated with R. prowazekii antigen (ET-S) or from leukocytes from non-typhus immune subjects non-specifically stimulated with PHA (PHA-S), or uninfected host cells, mostly F-1000 human fibroblasts, was complex and similar.

a. Clumping of treated cells in suspension. F-1000 human fibroblasts, either pre-treated with ET-S or PHA-S prior to trypsination from growth flasks or incubated in the presence of the supernatant fluids from stimulated leukocytes, showed a distinct tendency to aggregate or clump in the suspended cell infection system. The observation was made with considerable regularity on numerous occasions and, in this system at least, suggests that exposure to leukocyte supernatants results in some change in host cell surface that enhances clumping and this, therefore, constitutes one observable change induced in rickettsial host cells in which both antirickettsial and cytotoxic actions are expressed. In addition to its theoretical importance as a possible clue to an alteration of host cell which might eventually be correlated with the antirickettsial and cytotoxic mechanisms, this clumping caused some practical, technical problems because the quantitative relationships established for the suspended cell system for infection of cells with rickettsiae depends upon monodispersed cells settling freely under the influence of gravity. Hence, in studies in which infection

rate was critical (see below), it was necessary either to carry out experiments under conditions which minimized the clumping effect or to employ the stationary host-cell-sedimenting rickettsia system (Wisseman and Waddell, in preparation).

b. Effect of leukocyte supernatants on uninfected cells in microtiter cytotoxicity assay system. Two different kinds of action of active leukocyte supernatants were observed on uninfected cells in the microtiter "cytotoxicity" assay system, depending upon the kind of exposure.

(1) Cytostatic action in "post-treated" uninfected cells. When the supernatants were present continuously during a cytotoxicity type assay of uninfected cells corresponding to the "post-infection treatment" situation, there was a consistent average tendency to cause cytostasis, rarely very slight apparent loss of cells. This latter effect appeared to be somewhat more prominent with the PHA-S than with the ET-S, but no systematic quantitative study was made of this possible phenomenon. Significantly, no cytotoxic action was observed with L-929 cells, which have been employed extensively in assays for tumoricidal lymphotoxic activity (6) (See Figure 3).

(2) Growth stimulatory effect on "pre-treated" uninfected cells. In contrast, when the uninfected F-1000 cells were pre-treated for about 18 hrs with stimulated leukocyte supernatant fluids, then distributed to the wells of the microtiter plates as for a cytotoxicity assay and were subsequently incubated in the presence of normal tissue culture medium without leukocyte supernatant, a distinct growth stimulatory effect was observed (Figure 3). Various growth stimulatory actions have been described by others in the supernatant fluids of stimulated leukocytes.

Thus, supernatant fluids from stimulated leukocytes, whether typhus-immune stimulated by R. prowazekii antigen or non-immune stimulated with PHA, had some effects upon uninfected host cells, depending upon the conditions and kind of assay system employed. None of these, however, were comparable to the effects described in more detail below on rickettsia-infected cells and the degree to which they affected the detection of effects in rickettsia-infected cells was minimized by comparison with appropriate controls.

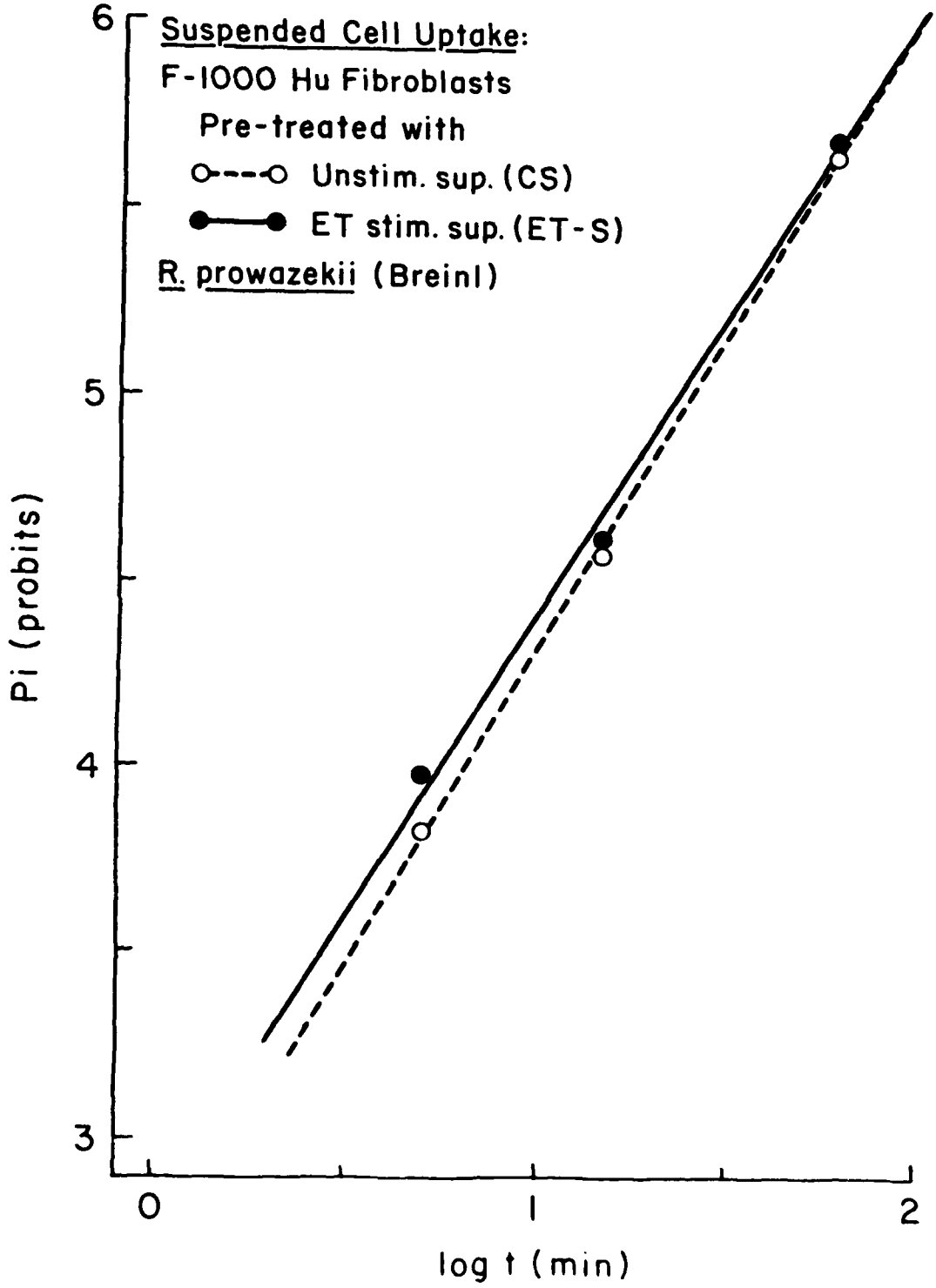
5. Analysis of effects of leukocyte supernatant fluids on R. prowazekii (Breinl)-infected F-1000 cells. Having established in the preceding control studies (a) that neither ET-S nor PHA-S supernatants exert a direct rickettsiacidal action on R. prowazekii (Breinl), (b) that assay systems can discriminate clearly between a distinct intracellular antirickettsial action and a cytotoxic action on infected cells and (c) that, although both types of supernatant have very definite effects upon uninfected host cells, these can easily be distinguished from the effects observed on infected cells, the actions of specific (ET-S) and non-specific (PHA-S) leukocyte supernatants on R. prowazekii (Breinl) infection of F-1000 fibroblasts was examined in greater detail.

FIGURE 3

Effect on Uptake of R. prowazekii (Breinl) by F-1000 Human Foreskin Fibroblasts Pre-treated for 18 h with Supernatant Fluids of Unstimulated and R. prowazekii-Stimulated Human Typhus-Immune Leukocytes: A Kinetic Study.

Note that pre-treatment of host cells with an active supernatant under conditions which permitted expression of intracellular antirickettsial action (see Figure 2) failed to influence significantly the capacity of R. prowazekii to infect them.

FIGURE 3


Suspended Cell Uptake:
F-1000 Hu Fibroblasts
Pre-treated with
Unstim. sup. (CS)
ET stim. sup. (ET-S)
R. prowazekii (Breinl)
Pi (probits)
6
5
4
3
0
1
2
log t (min)

a. Influence of pre-treatment of host cells with active leukocyte supernatant fluids on rickettsial uptake. The effect of pre-treatment of host cells on the uptake rate of untreated rickettsiae was next examined. In a study performed on two separate occasions in which great care was taken to assure maximal dispersion of host cells, no effect of pre-treatment of F-1000 cells with leukocyte supernatants was seen on the rate of uptake of untreated R. prowazekii in the suspended cell system (Figure 4). However, in many routine experiments in which pre-treated host cells were infected wth R. prowazekii along with appropriate controls (control medium and unstimulated leukocyte supernatant fluids), the per cent cells infected (p_i) was consistently lower in the treated cells. The most likely explanation lies in the distortion of uptake kinetics by the clumping tendency induced by active leukocyte supernatants. However, without a more exhaustive, quantitative study in which all factors are adequately controlled and uptake efficiency is calculated on the basis of successful penetrations per collision (Wisseman and Waddell, in preparation), we cannot categorically state the pre-treatment of host cells has no discernible effect on uptake rate. Nevertheless, it is clear that F-1000 human fibroblasts pre-treated with ET-S or PHA-S can be infected with a relatively high degree of efficiency in comparison with untreated cells and that the major effects described in this communication are not the result of a drastic reduction in infection rate.

b. Intracellular antirickettsial action of stimulated leukocyte supernatant fluids (Figures 5 and 6).

(1) Numerous replicate experiments, in which F-1000 cells, pre-treated with either ET-S or PHA-S, were infected with R. prowazekii (Breinl) and then observed microscopically for intracellular growth of the rickettsiae in Giménez-stained preparations. A reproducible pattern, illustrated in Figures 5 and 6, was observed with both types of stimulated leukocyte supernatants. The major features are as follows:

(a) In F-1000 cells pre-treated with control medium (CM) or unstimulated leukocyte supernatant (CS), the rickettsiae underwent an uninhibited growth cycle as previously established in CE cells. No inhibitory effect was observed.

(b) In F-1000 cells pre-treated with either ET-S or PHA-S and then infected with R. prowazekii (Breinl), a reproducible antirickettsial action was regularly observed. Thus, there was a rapid fall in the per cent cells infected (p_i) in the first 48 h, with little lag. The average number of rickettsiae per cell (N) usually reflected this antirickettsial action closely but on occasion was distorted by the fact that in a minority of cells relatively unrestricted growth of rickettsiae occurred (N_i). Thus, in the majority of pre-treated, infected cells, rickettsiae demonstrable by the Giménez stain did not grow and actually disappeared rapidly.

(c) However, a few cells appeared unaffected and in these rickettsial growth was essentially unrestricted, suggesting a certain variability in the host cell population in susceptibility to antirickettsial action of stimulated leukocyte supernatant fluids. In some experiments

FIGURE 4

Effect of Supernatant Fluid From Control and Both Specifically Stimulated Human Typhus-Immune Human Leukocytes (ET-S) and Non-Specifically Stimulated Human Typhus Non-Immune Human Leukocytes on Uninfected F-1000 Human Foreskin Fibroblasts in Microtiter Cytotoxicity Assay System.

Note cytostasis when cells were incubated continuously in the presence of stimulated leukocyte supernatant fluids (Post-treatment) (more apparent at 72 h in other experiments) and growth stimulatory effect when cells were pre-incubated with stimulated leukocyte supernatant (ET-S), plated into microtiter wells and incubated in the presence of tissue culture medium alone.

FIGURE 4

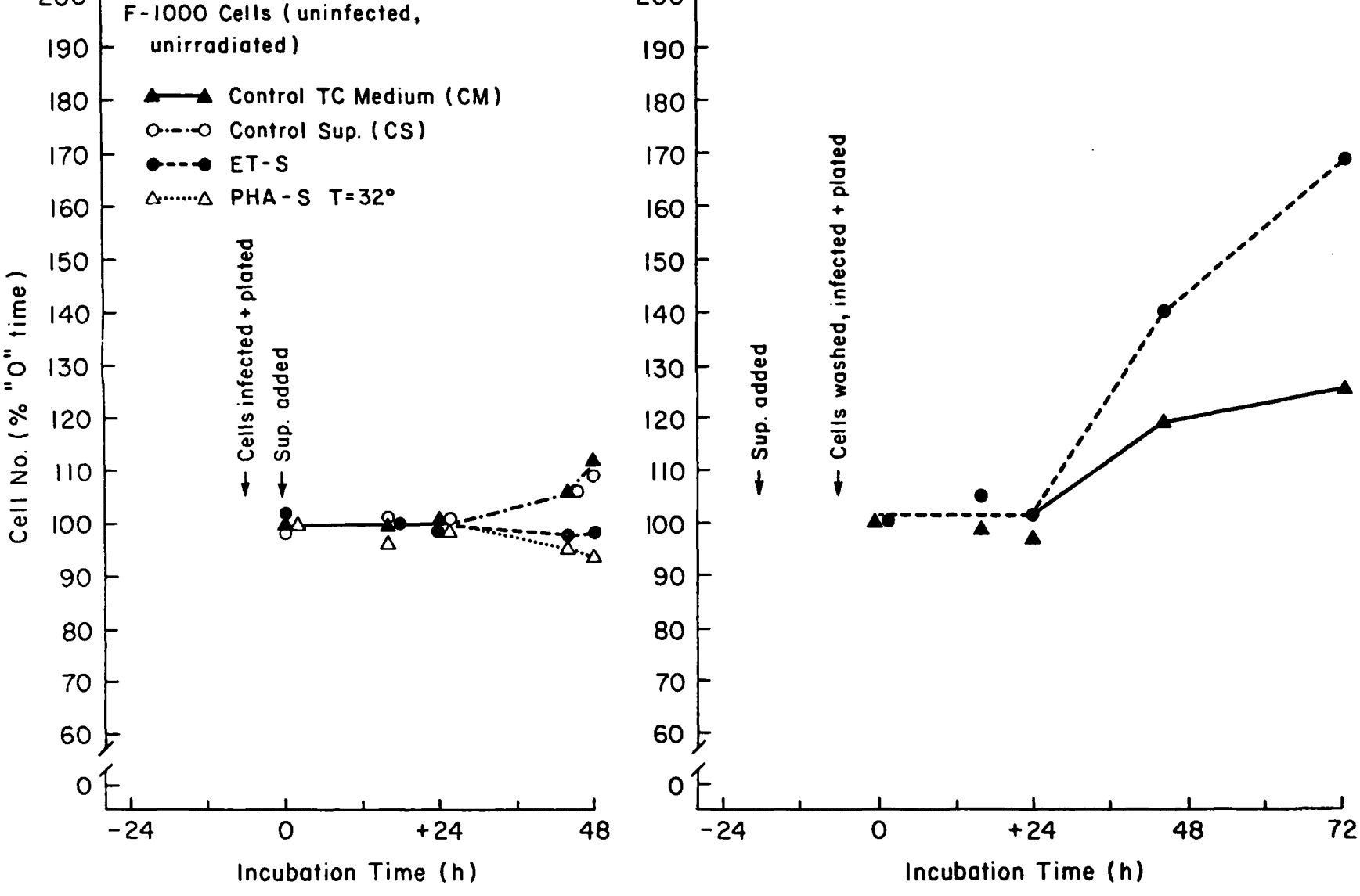

Post-Treatment
F-1000 Cells (uninfected, unirradiated)
Control TC Medium (CM)
Control Sup. (CS)
ET-S
PHA-S T=32°
Cells infected + plated
Sup. added
Cell No. (% "0" time)
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
0
-24
0
+24
48
Incubation Time (h)
Pre-Treatment
Sup. added
Cells washed, infected + plated
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
0
-24
0
+24
48
72
Incubation Time (h)

FIGURE 5

Antirickettsial, but not Cytotoxic, Action on F-1000 Human Foreskin Fibroblasts Pre-treated for 18 h with Supernatant Fluids from R. prowazekii-Stimulated Human Typhus-Immune Leukocytes before Infection with R. prowazekii (Breinl) in Assay Systems Specifically Designed for Each Effect.

FIGURE 5

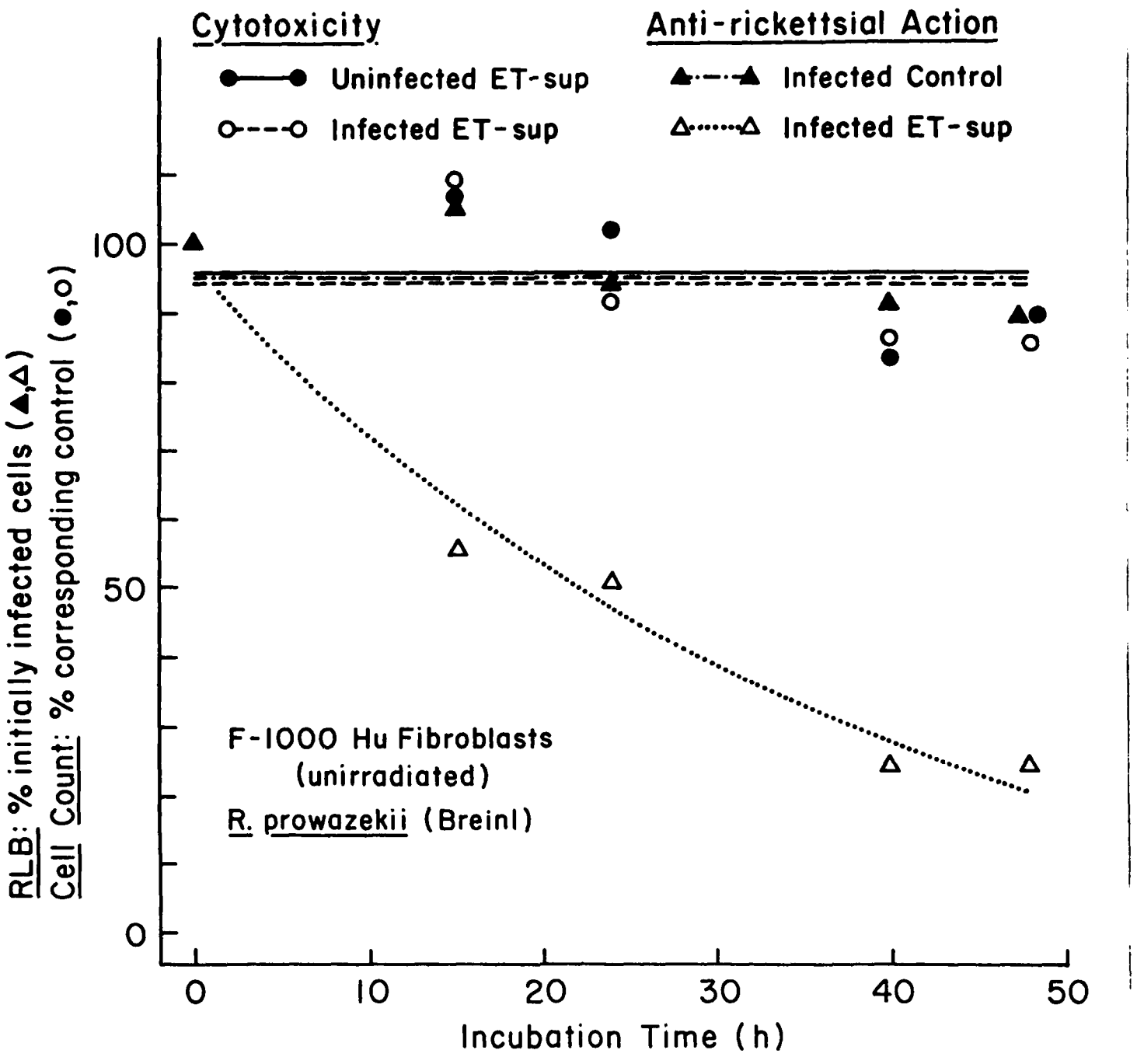
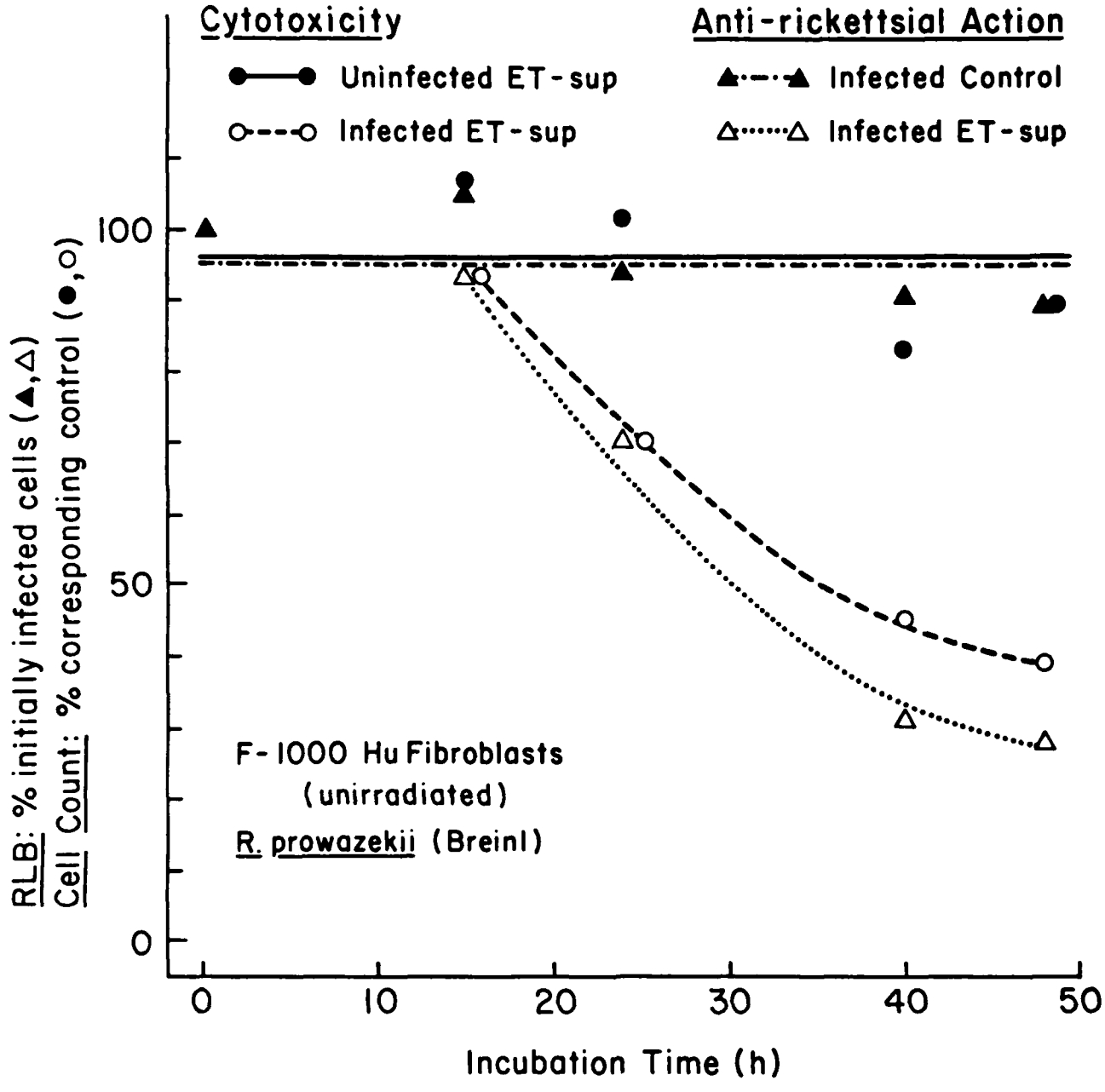

Cytotoxicity
Uninfected ET-sup
Infected ET-sup
Anti-rickettsial Action
Infected Control
Infected ET-sup
RLB: % initially infected cells (▲,△)
Cell Count: % corresponding control (●,○)
100
50
0
F-1000 Hu Fibroblasts
(unirradiated)
R. prowazekii (Breinl)
0
10
20
30
40
50
Incubation Time (h)

FIGURE 6

Both Antirickettsial and Cytotoxic Action of Supernatant Fluid from R. prowazekii-Stimulated Human Typhus-Immune Leukocytes Added to F-1000 Human Foreskin Fibroblasts 6 h after Infection with R. prowazekii (Breinl) (Post-Treatment) in Assay Systems Specifically Designed for Each Effect.

FIGURE 6


Cytotoxicity
Uninfected ET-sup
Infected ET-sup
Anti-rickettsial Action
Infected Control
Infected ET-sup
RLB: % initially infected cells (▲,△)
Cell Count: % corresponding control (●,○)
100
50
0
F-1000 Hu Fibroblasts
(unirradiated)
R. prowazekii (Breinl)
0
10
20
30
40
50
Incubation Time (h)

carried beyond 48 h, there appeared to be an "escape" phenomenon in which all parameters of rickettsial growth increased. It is not known at this time if previously inhibited and unstainable rickettsiae recovered from the effects of inhibition within leukocyte treated cells or if the infection sustained in the small numbers of relatively unaffected cells began to spread through normal mechanisms to involve cells which by this time had lost their capacity to restrict rickettsial proliferation.

(2) Fate of intracellular rickettsiae within cells pre-treated with stimulated leukocyte supernatants. Because it is remotely conceivable that the loss of visible, stained rickettsiae in pre-treated cells, described above, could be the result of altered staining characteristics rather than loss of viable rickettsiae, two types of experiments have been initiated neither of which is in sufficient state of completion at this time to warrant predictions as to results. These experiments are as follows:

(a) Disruption of infected control and treated cells at intervals after infection and titration of viable rickettsiae by the plaque method.

(b) Sequential electron-microscopic observations of the morphological changes in the rickettsiae.

c. Cytotoxic action of stimulated leukocyte supernatants on R. prowazekii (Breinl)-infected F-1000 cells (Figures 5 and 6). Experiments were carried out in parallel with the antirickettsial studies described above in "b", using the microtiter technique to measure the cytotoxic action of stimulated leukocyte supernatants on R. prowazekii (Breinl)-infected F-1000 human fibroblasts. The impressions gained from the preliminary studies described in "1" above were confirmed by application of quantitative assays to measure cytotoxicity specifically. Thus, when F-1000 cells were pre-treated with active supernatants (See Figure 6), then infected and distributed to microtiter plates and incubated in the absence of supernatants, no loss of cells, i.e., no cytotoxicity, was detected even though a pronounced antirickettsial action, without significant lag, was readily apparent in parallel slide chamber cultures. In contrast, when active supernatants were added to microtiter wells containing cells already infected (Figure 5), a pronounced loss of cells, i.e., cytotoxicity, was observed after a lag of 12-24 h, at about the same time that an apparent antirickettsial action was observed in parallel slide chamber cultures, as described in the original observations in "1". Essentially identical results were obtained regardless of whether immunologically specific (ET-S) or non-specific (PHA-S) leukocyte supernatant fluids were used. These same supernatant fluids had no such action upon uninfected cells. Supernatants from unstimulated human leukocytes had no such cytotoxic action.

Thus, the cytotoxic action of supernatants from stimulated human leukocytes specific for R. prowazekii-infected F-1000 Hu fibroblasts was confirmed. No such action was demonstrable with these same supernatants on similarly infected chicken embryo fibroblasts.

6. Host cell specificity of antirickettsial and cytotoxic actions of stimulated human leukocyte supernatant fluids. Preliminary experiments described in "1" indicated that stimulated human leukocyte supernatants exerted antirickettsial and cytotoxic actions on diploid human fibroblasts (WI-38 and F-1000) but not on secondary chicken embryo fibroblasts. This suggested a host cell specificity, not unlike that of the interferon systems. To test the range of host cell specificity exhibited by supernatants derived from human leukocytes, we have embarked on a systematic testing for both antirickettsial and cytotoxic actions in a selected variety of primary, continuous diploid and transformed cells of human, primate, mouse and avian origin. The study is not yet complete, but the results which are available, recorded in Table 4, give preliminary support for considerable host cell specificity for both ET-S and PHA-S. Thus far, no non-human cell has permitted either action to be expressed. It remains to be determined if human origin transformed or malignant cells, monkey primary or diploid and primary mouse embryo fibroblasts will permit expression of these effects to permit some interpretation of the importance of host cell species and normal or transformed state in the expression of these effects. Of special significance, however, is that the L-929 mouse fibroblast did not permit the demonstration of either type of effect. Lines of the L-929 cell have been found to be especially sensitive to lymphotoxins. We are currently attempting to acquire the "alpha" line of the L-929 mouse fibroblast, which is especially sensitive to lymphotoxins, in the attempt to detect classical lymphotoxins in these active leukocyte supernatants.

7. Microbial specificity of antirickettsial and cytotoxic actions of stimulated human leukocyte supernatant fluids. A systematic study of the capacity of ET-S (R. prowazekii system) and PHA-S to exert both antirickettsial and cytotoxic action on F-1000 human fibroblasts infected with different members of the typhus, spotted fever and scrub typhus groups of rickettsiae was also initiated. These studies, though not complete, are in an advanced state of execution and are summarized in Table 5. All tests were performed with a single ET-S and a single PHA-S preparation. Three distinct patterns were detected for both ET-S and PHA-S systems, according to the rickettsia tested.

a. Both antirickettsial and cytotoxic actions expressed. F-1000 cells infected with R. canada or, to varying degrees, strains currently classified as R. prowazekii showed both antirickettsial and cytotoxic actions induced by both ET-S and PHA-S.

It is of interest to note that, while all strains of R. prowazekii were associated with a strong cytotoxic action, not all strains were equally sensitive to the antirickettsial action. Thus, antirickettsial action was pronounced with the Breinl strain, an old laboratory strain with a long passage history following its isolation from a patient with epidemic typhus in Europe, and the Burundi strain, a recent isolate with short passage history from a typhus fever patient in central Africa. The effect was more variable with the E strain, an attenuated variant with long passage history of a strain isolated from a typhus fever patient in Madrid. Only slight to negligible antirickettsial action was observed with a strain recently isolated

Table 4 Host cell specificity of antirickettsial and cytotoxic actions of "specific"[1] and "non-specific"[2] human leukocyte supernatants on R. prowazekii (Breinl) infection.

Host Cells			Type of Action			
Origin/ Designation	Cell Type	State[5]	Anti-Rickettsial[3] ET-S[1]	Anti-Rickettsial[3] PHA-S[2]	Cytotoxic[4] ET-S[1]	Cytotoxic[4] PHA-S[2]
Homo Sapiens						
WI-38	Embryonic Lung Fibroblast	D	+	+	ND	ND
F-1000	Foreskin Fibroblast	D	+	+	+	+
HeLa	Cervical Carcinoma Epithelial-like	T	ND	ND	ND	ND
Macrophage	Monocyte-derived	P	--- see text ---			
Endothelial	Umbilical Vein	P	--- see text ---			
Sub-human Primate						
DBS-FCL-1	Fetal lung fibroblast-like	D	ND	ND	ND	ND
VERO	Normal Af. Green Monkey Kidney fibroblast-like	T	-	-	-	-
LLC-MK_2	Normal Rhesus Monkey Kidney Epithelial-like	T	-	-	-	-
Rodent (Mus)						
Mouse embryo	Fibroblast	P	ND	ND	ND	ND
L-929	Mouse connective tissue fibroblast-like	T	-	-	-	-
Avian						
Chicken embryo	Fibroblast	P	-	-	-	-

1 ET-S: Supernatant from R. prowazekii-stimulated leukocytes from human subject convalescent from R. prowazekii infection.

2 PHA-S: Supernatant from PHA-stimulated leukocytes from non-immune subject.

3 Anti-Rickettsial Action: rickettsiastatic or rickettsiacidal action in cells pre-treated with leukocyte supernatant and removed prior to infection.

4 Cytotoxic: loss of infected cells caused by leukocyte supernatants added 6 h after infection and left in contact with infected cells.

5 D = diploid; T = transformed, malignant or polyploid; P = primary or secondary cells from indicated tissue.

Table 5 Differential antirickettsial and cytotoxic actions of "specific"[1] and "non-specific"[2] human leukocyte supernatants on various rickettsial species and strains in F-1000 human fibroblast cell cultures

Species/ Strain	Type of Action			
	Anti-Rickettsial[3]		Cytotoxic[4]	
	ET-S[1]	PHA-S[2]	ET-S[1]	PHA-S[2]
R. prowazekii				
Breinl	+	+	+	+
Burundi	+	+	+	+
E	+ to ±	+ to ±	+	+
Fl. sq.	± to -	± to -	+	+
R. mooseri				
Wilmington	-	-	+	+
Ethiopian	ND	ND	ND	ND
R. canada	+	+	+	+
R. rickettsii				
Sheila Smith	-	-	+	+
R. tsutsugamushi				
Gilliam	-	-	-	-
JC-472 (Karp-like)	-	-	-	-

1 *ET-S*: Supernatant from *R. prowazekii*-stimulated leukocytes from human subject convalescent from *R. prowazekii* infection.

2 *PHA-S*: Supernatant from PHA-stimulated leukocytes from non-immune subject.

3 *Anti-Rickettsial* Action: rickettsiastatic or rickettsiacidal action in cells.

4 *Cytotoxic* Action: loss of infected cells caused by leukocyte supernatants added 6 h after infection and left in contact with infected cells.

in this country from flying squirrels by Bozeman et al. Additional studies are being performed with the last two agents. The significance of this variation is unknown. It is perhaps significant that strong antirickettsial action was noted with virulent strains isolated from human typhus infections.

R. canada, which also was associated with both antirickettsial and cytotoxic actions, though currently included in the typhus group on the basis of serology, is shown in another section of this report to differ substantially from R. prowazekii, R. mooseri and R. rickettsii in degree of DNA hybridization, genome size and PAGE protein profiles and may represent a new subgroup under the genus Rickettsia.

b. Cytotoxic action but not antirickettsial action expressed. A distinct cytotoxic action by ET-S and PHA-S was noted with cells infected with R. mooseri or R. rickettsii, but with neither organism was antirickettsial action expressed in a detectable or measurable manner. Recent isolates of both will be tested in the future. One important difference between these two species and R. canada and R. prowazekii lies in the fact that they produce rapidly spreading infections in cell cultures, with organisms escaping from host cells from early hours after initial infection. Whether this property inhibits expression or measurement of the antirickettsial action is unknown, but requires additional studies.

c. Neither antirickettsial nor cytotoxic actions expressed. Neither of two serotypes of R. tsutsugamushi, the Gilliam strain and a Karp-like isolate from the Sialkot region of Pakistan, were susceptible to either antirickettsial or cytotoxic actions of ET-S or PHA-S in the F-1000 fibroblasts. This is especially interesting because Nacy and Osterman (11) and Nacy and Meltzer (12) have shown that mouse lymphokines cause mouse peritoneal macrophages to express some kind of antirickettsial action on the Gilliam strain. In contrast, the factor(s) that we are studying in a human system, which exert both antirickettsial and cytotoxic actions with other rickettsiae in "somatic", non-phagocytic cells, have no effect on R. tsutsugamushi.

These range-finding experiments have revealed an interesting pattern of expression of antirickettsial and cytotoxic actions of stimulated leukocyte supernatants with representatives of different groups of organisms within the genus Rickettsia. With the exception of R. tsutsugamushi strains, the cytotoxic action was detected with all other groups of the genus. Antirickettsial action was more restricted, varying even with strains of the same species. These results suggest, but do not prove, independent mechanisms and entities for the two types of action. In no instance tested to date has antirickettsial action been detected without demonstrable cytotoxic action, but the converse is not true.

8. Expression of action of stimulated human leukocyte cell supernatants on rickettsiae in human blood monocyte-derived macrophages and human umbilical vein endothelial cells. Although the action of stimulated human leukocyte supernatants on rickettsial infection, obligate intracellular bacterial parasites, in human fibroblasts ("somatic" cells) establishes a new, unique

phenomenon of considerable potential importance to immune mechanisms of such parasites, the main target cell of rickettsial infection in Man is the endothelial cell of small blood vessels and the macrophage is a potentially important effector cell. Studies have been initiated to explore the actions of stimulated leukocyte supernatants on rickettsial infection in these two cells in vitro. They are still in preliminary stages.

a. Human monocyte-derived macrophages. Previous work in these laboratories has established the fact that human monocyte-derived macrophages alone in vitro are incapable of restricting the growth of either R. mooseri or R. prowazekii in the absence of opsonizing pre-treatment of rickettsiae with immune serum (2-5). Studies, using electron microscopic techniques, are described elsewhere in this report which show that, in the absence of immune serum, R. prowazekii which are phagocytized by macrophages in culture produce, within minutes, a defect in the vacuolar membrane which occurs before any significant lysosomal fusion takes place. This permits the rickettsia to escape into the cytoplasm where it multiplies in an unrestricted fashion. On the other hand, it was previously shown that R. mooseri and R. prowazekii, which had first been treated with immune serum, were opsonized and ingested at a greater rate than rickettsiae exposed to non-immune serum and were progressively destroyed by the macrophages. The ultrastructural study described elsewhere in this report demonstrates that immune serum-opsonized R. prowazekii were retained within the phagocytic vacuole, very rapid lysosomal fusion occurred, again within minutes, and the retained rickettsiae underwent rapid morphologically apparent destruction. Thus, immune serum has no direct rickettsiacidal action (17) and is incapable of restricting rickettsial (R. mooseri) replication locally in tissues, as demonstrated in these laboratories in the skin of guinea pigs (8-10) or the spleen of mice (elsewhere in this report). Macrophages, normal or activated, do not restrict R. mooseri replication in mouse spleen in the absence of rickettsial immune serum (described elsewhere in this report). However, there are reasons to believe that the particular models employed, though they tested the practical in vivo situation, may not have constituted an adequate test for the capacity of "activated" macrophages to destroy rickettsiae. In this situation, many of the rickettsiae, in the absence of immune serum, may simply have infected other cell types without coming into contact with macrophages. Hence, it is possible that macrophages activated by lymphokines in the classical scheme of cell mediated immunity may have enhanced capacity to destroy typhus rickettsiae without the aid of specific antibodies. Indeed, Nacy and Osterman (11) and Nacy and Meltzer (12) have described enhanced capacity of mouse peritoneal macrophages to restrict the growth of the Gilliam strain of R. tsutsugamushi when treated with stimulated supernatants from immune spleen cells.

We have initiated studies in which the capacity of human peripheral monocyte derived macrophages in culture, pre-treated with stimulated human leukocyte supernatants (ET-S and PHA-S), to restrict rickettsial multiplication is examined. In preliminary experiments in which macrophages were pre-treated for about 18 hours as in the established antirickettsial action assay described above, we have found that R. prowazekii (Breinl) growth was restricted in a manner similar to that observed in human fibroblasts but that R. tsutsugamushi (Gilliam) growth was not restricted, similar to the observations described

above in fibroblasts and contrasting with the murine system described by Nacy et al. (11, 12). Further studies are in progress. If these preliminary results are confirmed, it would appear that the antirickettsial factors in leukocyte supernatants with which we are working may not be acting to restrict rickettsial growth in human macrophages by the classical CMI mechanism of "angry" macrophages and that this system differs from the murine system operative on R. tsutsugamushi as described by Nacy et al. (11, 12).

b. Human umbilical vein endothelial cells. In collaboration with Dr. Ances of the Department of Obstetrics and Gynecology and Dr. David Silverman of this Department, we have been attempting to propogate human umbilical cord endothelial cells in cell culture and to adapt them to our assay systems. Endothelial cells are notoriously difficult to establish in number in long-term cultures and to manipulate.

We have made some progress in establishing primary monolayers of umbilical cord endothelial cells but have not yet worked out optimal conditions for the regular long-term survival of the majority of endothelial cells following manipulation of the primary cell monolayers in various experimental procedures. However, it has been possible to carry out a preliminary experiment on the effects of pre-treatment of endothelial cells with ET-S and PHA-S on the fate of R. prowazekii (Breinl). This experiment clearly showed the same kind of antirickettsial action demonstrated in fibroblasts. Thus, with some refinements in cell culture technology, we should be able to carry out critical studies in cultures of the main "target" cell for rickettsial invasion and growth in typhus fever, spotted fever and scrub typhus fever of Man.

SUMMARY

1. Two new mechanisms for the expression of CMI in typhus infection in Man appear to have been confirmed and partially characterized in vitro namely, (1) an intracellular antirickettsial action and (2) a cytotoxic action on infected cells.

2. Both mechanisms are mediated through the supernatant fluids of human leukocytes that have been stimulated by either immunologically specific mechanisms (R. prowazekii antigen with R. prowazekii immune leukocytes) or non-specifically by a T-cell mitogen (PHA). This suggests that the mediators represent final common pathways.

3. The active leukocyte supernatant fluids have no demonstrable action on the infectivity or viability of extracellular rickettsiae. The effects seem to be mediated through the host cell.

4. Both mechanisms operate on "somatic cells", i.e. non-phagocytic cells. The antirickettsial action is also expressed in monocyte derived macrophages in vitro, probably by mechanisms which do not involve the activation phenomenon of classical anti-Tb or Listeria CMI, and in human umbilical cord endothelial cells.

5. Both types of action appear to be highly host cell species specific. To date, they have been demonstrated only with cells of human origin.

6. The antirickettsial action was demonstrable, without attendant cytotoxicity, in host cells that had been treated with active leukocyte supernatant fluids prior to infection and that were incubated in the absence of leukocyte factors after infection. In this regard, the factor was similar to interferon, but one very active supernatant which was tested by Dr. Ed Havell of the Trudeau Institute for the presence of interferon in two sensitive systems was devoid of significant interferon action.

7. Cytotoxicity was expressed when previously infected cells were incubated continuously in the presence of active leukocyte supernatant fluids. Under these conditions, uninfected cells showed cytostasis but no cytotoxicity.

8. Antirickettsial action and cytotoxicity showed a tendency to be expressed independently with different rickettsial species. Thus, cytotoxicity was observed in cells infected with R. prowazekii, R. mooseri, R. canada and R. rickettsii, but not with R. tsutsugamushi. Antirickettsial action was demonstrable with R. prowazekii and R. canada, but not with R. mooseri, R. rickettsii or R. tsutsugamushi. The tendency of R. mooseri and R. rickettsii to escape from infected cells early in the course of infection and to spread to other cells may have had an influence on both the opportunity for expression of antirickettsial action and on the accuracy of the assay method. The degree of antirickettsial action in R. prowazekii appear to vary with the strain, being most pronounced with the virulent Breinl (an old lab strain) and the Burundi strain (a recent isolate from a typhus patient), somewhat less with the attenuated E strain (a mutant from a strain isolated from a typhus patient) and least with a Flying Squirrel strain.

Obviously, much more work needs to be done to identify the cells which produce these factors, to characterize the factors themselves, to determine mode of action, to relate them to other soluble factors (lymphokines of various types, etc.) and so on. Nevertheless, we believe that we have accumulated a substantial body of evidence in support of the existence in Man of unique CMI mechanisms which can control the intracellular replication of at least some of these obligate intracellular parasitic bacteria in "somatic", "target" cells, as occur in tissues infected with rickettsiae, and can selectively damage such infected cells by mechanisms which do not involve activated macrophages, although macrophage activation does occur (vide infra), and under conditions where antibody has no demonstrable effect either in vivo or in vitro. Such actions would be consistent with our observations in guinea pigs and mice on the control of rickettsial replication locally in tissues by immune T-lymphocytes under conditions in which neither antibody nor activated macrophages separately were effective. This does not detract from the concept that antibody-sensitized rickettsiae can be cleared and destroyed by macrophages.

B. Electronmicroscope studies of mechanisms by which virulent Rickettsia prowazekii (Breinl) circumvents the microbicidal action of human blood monocyte-derived macrophages in vitro and the morphological events which lead to the intraphagosomal destruction of immune serum-opsonized rickettsiae. (With Wm. A. Meyer) Previous studies in these laboratories (2-5), using light microscopy, established that, when either R. prowazekii or R. mooseri, incubated with non-immune human serum, were phagocytized in vitro by human blood monocyte derived macrophages, the rickettsiae grew in the cytoplasm as in non-phagocytic cells and destroyed the macrophages. However, when the rickettsiae were first incubated with human typhus-immune serum, the opsonized and phagocytized rickettsiae were destroyed. An early electron microscope study (1) of the phenomena with R. mooseri and human blood monocyte-derived macrophages showed that rickettsiae which had been incubated with non-immune serum prior to phagocytosis were soon found free in the macrophage cytoplasm, where they multiplied unbounded by a vacuolar membrane. In contrast, the rickettsiae which had been pre-treated with typhus-immune serum were retained in phagocytic vacuoles and underwent morphologic degradation. Ultrastructural details of these processes, however, were incomplete. Other studies (17; Krause and Wisseman, unpublished) had shown that immune serum with or without added complement and divalent cations, had no rickettsiacidal action and did not prevent infection of yolk sac cells in ovo or of chicken embryo fibroblasts in vitro.

The present study examines the rickettsia-immune serum-macrophage interaction by quantitative measurements of ultrastructural changes with respect to time, using human blood monocyte-derived macrophages whose secondary lysosomes were labeled with Thorotrast^{R} (colloidal thorium-dioxide) and the virulent R. prowazekii (Breinl). It compares the sequence of morphological events following phagocytosis of (1) glutaraldehyde-killed R. prowazekii, (2) viable R. prowazekii pre-incubated with non-immune human serum and (3) viable R. prowazekii pre-incubated with typhus-immune human serum.

The basic method was briefly as follows. Peripheral blood mononuclear cells were obtained from healthy typhus non-immune medical students by a modified Ficoll-Hypaque centrifugation technique. After 6 days in culture, an electron-dense marker (Thorotrast^{R}) was added to the macrophage cultures and allowed to concentrate in secondary lysosomes. Extracellular Thorotrast^{R} was removed. Appropriately treated R. prowazekii (Breinl) were added for 15 minutes and then washed away. A sample taken at this point for electron-microscopy was designated "0" time, even though it was recognized that the intracellular rickettsial population had been in residence for from 0 to 15 min. This represented a compromise which permitted practical numbers of rickettsiae to be phagocytized without causing macrophage cytotoxicity. Incubation was continued at 34^{o}C in TC medium. Additional samples were regularly taken at 10 min, 30 min and 6 h, and occasionally at other designated times. All were fixed and processed for examination by transmission electron microscopy. Ultrathin sections were examined for (1) location of rickettsiae within the macrophage, (2) integrity of the phagosomal membrane, (3) ultrastructural integrity of the rickettsiae and (4) whether or not thorotrast was present in phagosomes containing rickettsiae. A minimum

of 100 rickettsiae were examined and scored for each time point under each condition. Since these studies, though very advanced, are not yet complete, graphical representation of the time course of morphological events was not attempted for this report. However, the electron micrographs in Figures 7 through 10 illustrate clearly the key points of the following narrative description.

1. Glutaraldehyde-killed R. prowazekii. (Figure 7). These non-viable, non-antibody coated rickettsiae served as a control against which to guage other experiments. Initially, ultrastructurally normal rickettsiae were found within membrane-bound phagocytic vacuoles that contained no visible lysosomal products. However, even at "0" time, particles from Thorotrast^R-containing lysosomes were found within 22% of the vacuoles and occasionally lysosomes could be found fused with the phagosome in the process of discharging their Thorotrast-labeled contents. Lysosomal fusion, as evidenced by the presence of Thorotrast^R particles in rickettsia-containing phagosomes, was progressive. By 6 h, 94% of the phagosomes contained Thorotrast^R particles and many of the rickettsiae showed degenerative morphological changes.

2. Viable R. prowazekii pre-incubated with non-immune serum. (Figures 8 and 9). In marked contrast to the findings with glutaraldehyde-killed rickettsiae, phagocytized viable rickettsiae without antibody rapidly induced a defect in the phagosomal membrane which progressively enlarged, leaving the organisms free in the macrophage cytoplasm without evidence of lysosomal discharge. This process, already detectable at "0" time, was so rapid that at 30 min 70% of the organisms were already free in the cytoplasm unbound by a phagosomal membrane. These organisms multiplied in the macrophage cytoplasm, much as in non-phagocytic cells, virtually to fill the cytoplasm by 72 h, surrounded by electron lucent material presumed to be the slime layer or capsule.

When the rate of escape of rickettsiae from phagocytic vacuoles in this study was compared with the rate of lysosomal fusion with glutaraldehyde-killed rickettsiae (above) or antibody treated rickettsiae (below), it was apparent that the rate of escape was greater than the rate of lysosomal fusion. Thus, one mechanism by which R. prowazekii evades or circumvents the microbicidal action of the macrophage appears to be to escape from the vacuole before lysosomal fusion occurs. Another mechanism exhibited by some bacteria and protozoa (and perhaps C. burnetii, though this has not yet been examined) is active inhibition of lysosomal fusion. Unfortunately, the rate of escape of R. prowazekii from vacuoles was so rapid that we could not make definitive observations on this phenomenon. However, it is clear that the rickettsiae alter the phagosomal membrane in some manner to create a defect. It is conceivable that such an altered membrane may not fuse readily with lysosomes. Indeed, intact lysosomes were often seen very near a phagosome, occasionally in apparent contact with it or with the slime layer of the exposed rickettsia without showing membranal fusion or damage and Thorotrast^R particles were not seen free in the cytoplasm as evidence of lysosomal discharge or lysis. This matter remains to be clarified.

FIGURE 7

Phagocytosis of Glutaraldehyde-killed R. prowazekii (Breinl) by Human Monocyte-Derived Macrophages in Culture.

Symbols: R = rickettsia; L = Thorotrast-labeled secondary lysosomes; M = mitochondria. Bar = 500 nm

A. Rickettsia in membrane-bound phagosome at "0" time. No evidence yet of lysosomal fusion.

B. Thorotrast-containing secondary lysosome fusing with rickettsia-containing phagosome. 0 min.

C. Rickettsia-containing phagolysosome with abundant Thorotrast indicating extensive lysosomal fusion. 10 min.

D. Three Thorotrast-containing phagolysosomes with rickettsiae in advanced stages of morphological degradation. 6 h.

FIGURE 7

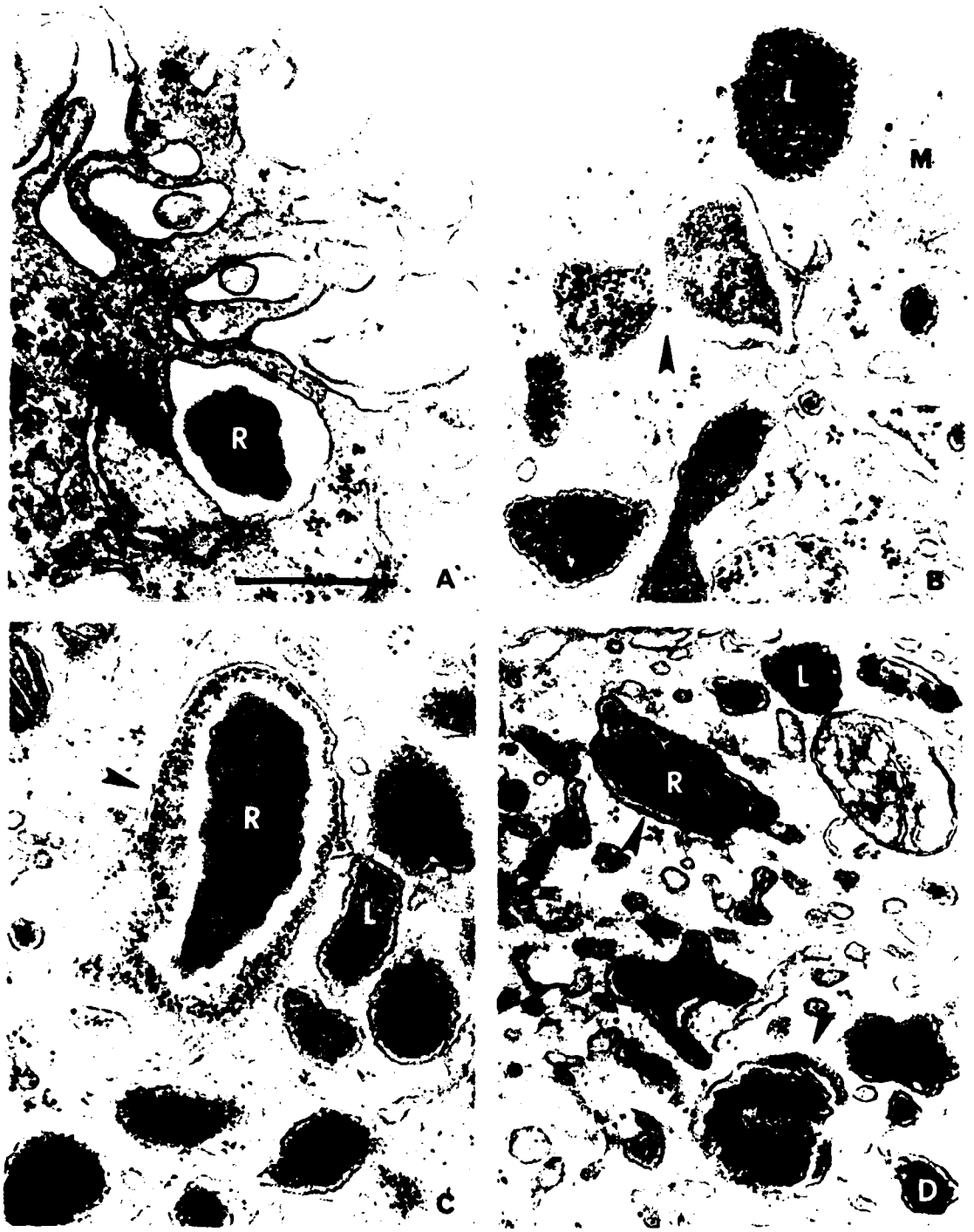


FIGURE 8

Escape of Viable Virulent R. prowazekii (Breinl) from Phagosomes of Human Monocyte-Derived Macrophages in the Presence of Non-Immune Human Serum.

A. Rickettsia in membrane-bound phagosome immediately after phagocytosis. No evidence of lysosomal fusion. About 0 min. Bar = 500 nm

B. Early defect in phagosomal membrane. Note clear zone, probably slime layer, about rickettsia. 0 min.

C. Advanced stage of phagosomal membrane destruction with rickettsia, still surrounded by slime layer, free in macrophage cytoplasm. No evidence of lysosomal fusion. About 10 min.

D. Macrophage showing abundant rickettsial growth in macrophage cytoplasm (this macrophage was not labeled with Thorotrast). Rickettsiae, not bound by membranes, surrounded by abundant electronlucent slime layer material. Bar = 5 μm

FIGURE 8

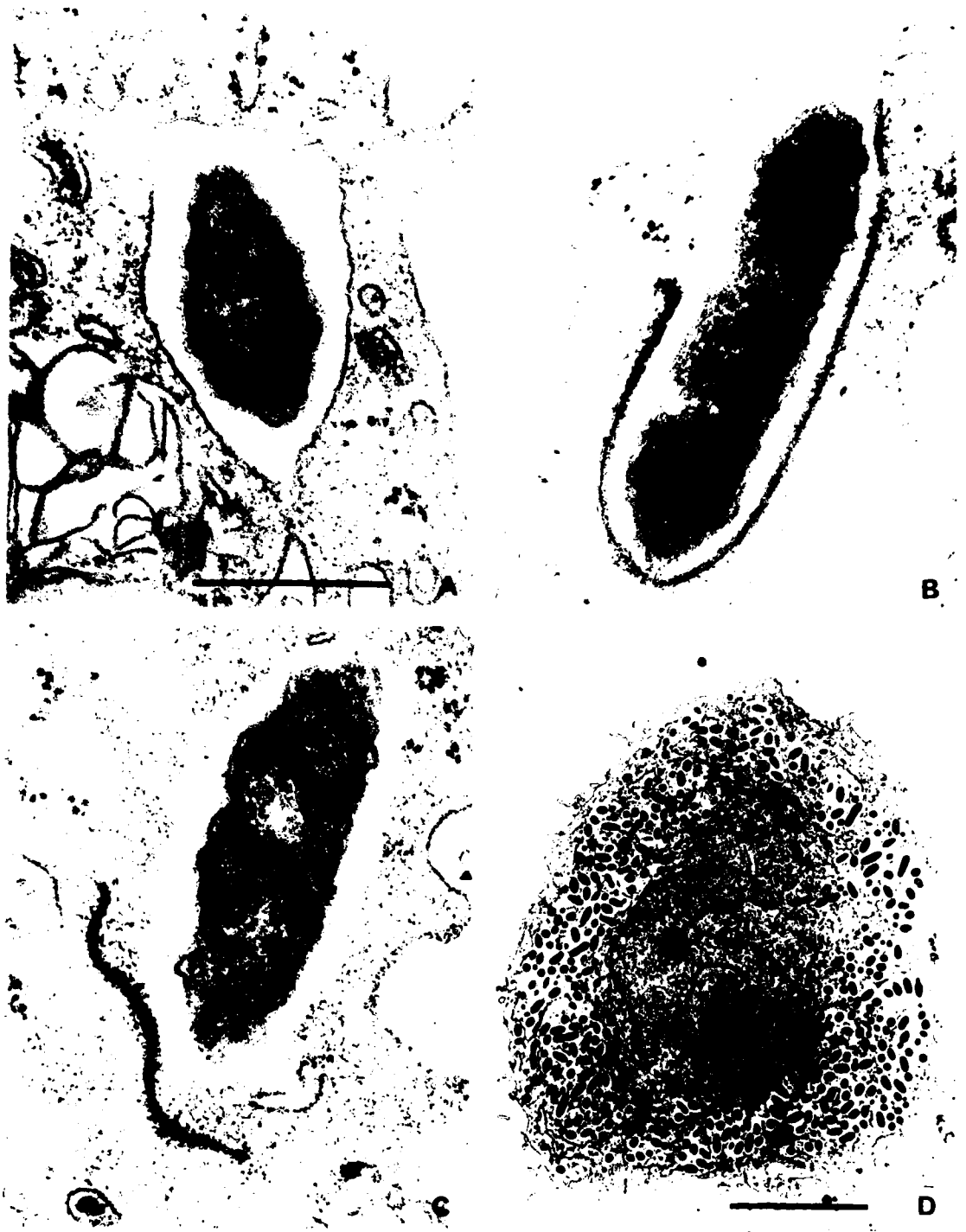


FIGURE 9a

Further Details of Escape of Viable Virulent R. prowazekii (Breinl) from Phagosomes in Human Monocyte-Derived Macrophages in Culture. Bar = 500 nm

Note the thickened, fuzzy recurved edges of the phagosomal membrane at the edge of the expanding defect. Note also absence of Thorotrast lysosomal tracer in phagosomes or free in cytoplasm and proximity of intact labeled secondary lysosomes to phagosomes (B) and free rickettsial slime layer (C).

FIGURE 9a

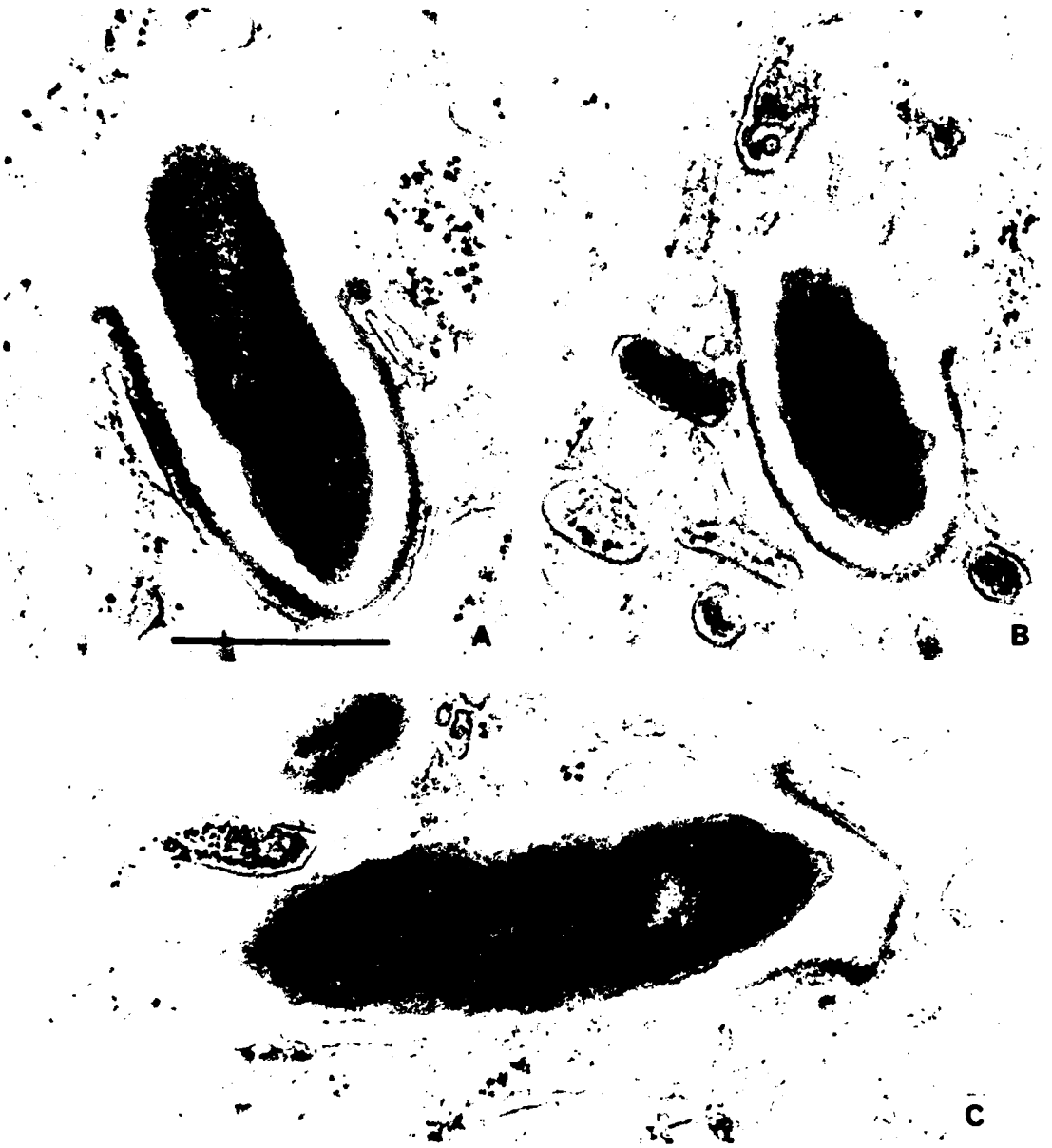
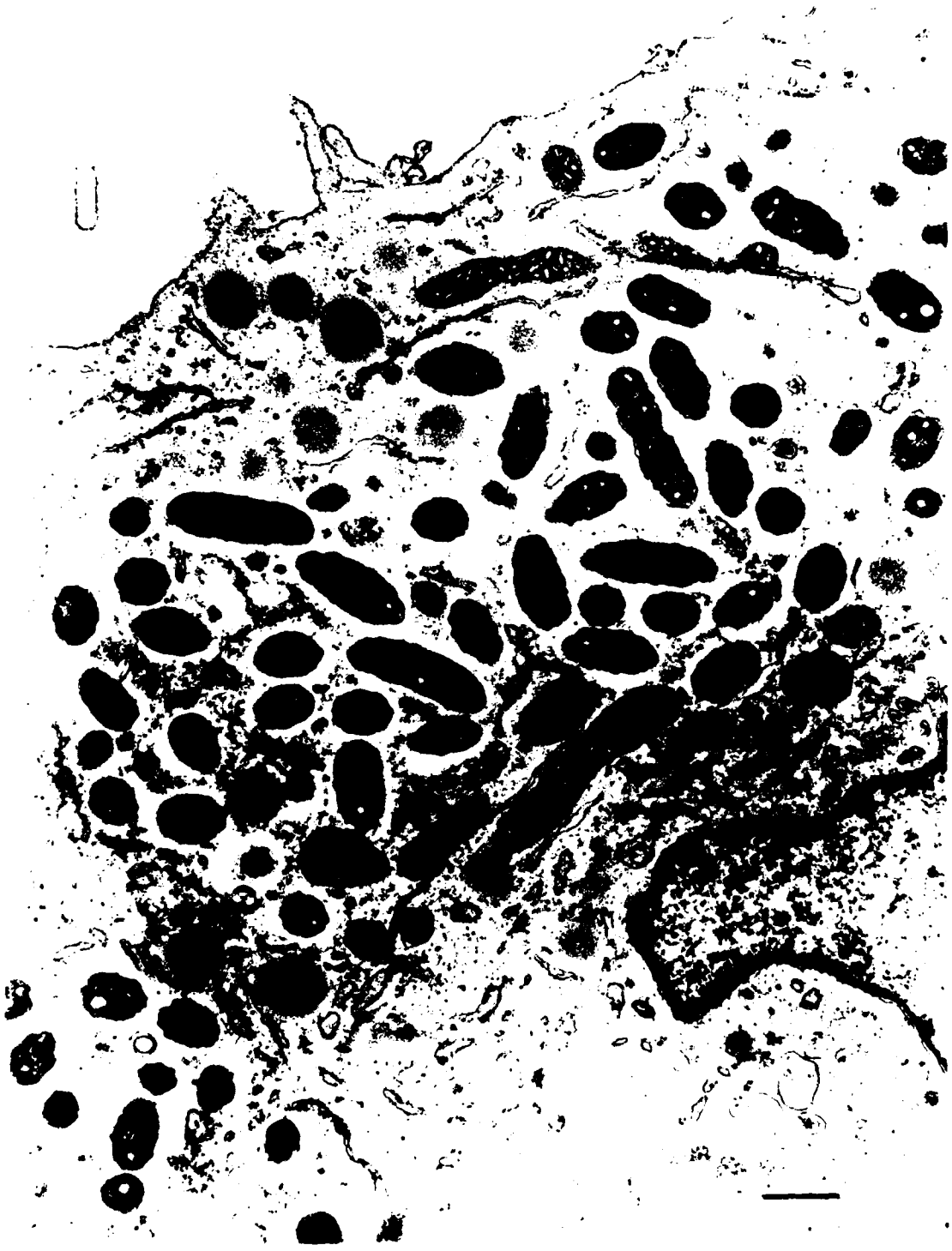


FIGURE 9b

Virulent R. prowazekii (Breinl) Growing in Cytoplasm of Human Monocyte-Derived Macrophage. 72 h. (Higher magnification of portion of Figure 8D) Bar = 500 nm

Note absence of membranes about rickettsiae, abundant electronlucent material around rickettsiae displacing cytoplasmic material and intact mitochondria adjacent to rickettsiae. Scattered, few ribosomes on endoplasmic reticulum.

FIGURE 9b



3. Viable R. prowazekii pre-incubated with typhus-immune human serum. (Figure 10). The sequence of morphological events which occurred within macrophages after phagocytosis of viable R. prowazekii pre-incubated with human typhus immune serum was very similar to that found with glutaraldehyde-killed rickettsiae and differed markedly from that seen with viable rickettsiae in the absence of immune serum. Avid phagocytosis was closely followed by rapid, marked and progressive fusion of lysosomes with rickettsia-containing phagosomes. This was accompanied by early, progressive morphological evidence of rickettsial damage in phagosomes containing lysosomal products. By 6 h the damage was so extensive that the organisms were difficult to identify. Only very rarely was an intact rickettsia found free in the cytoplasm, a finding consistent with previous light microscope studies which revealed the very rare cell in which rickettsial multiplication was evident.

Thus, the great majority of immune serum-treated viable R. prowazekii are retained within phagocytic vacuoles, are rapidly exposed to lysosomal enzymes and rapidly undergo morphological degradation. The precise mechanisms by which the organisms are killed are not yet known. The antiserum is not rickettsiacidal (17). R. prowazekii neither possesses catalase nor produces hydrogen peroxide (7). It remains to be determined if the myeloperoxidase-halide system is operative.

C. Failure of activated macrophages to control R. mooseri replication in mouse spleen in the absence of antibodies. (With A. Crist and J.R.Murphy). The in vitro studies of the preceding section with R. prowazekii and human macrophages suggest one mechanism by which typhus rickettsiae so efficiently circumvent innate host defense mechanisms with non-immune subjects that less than 10 organisms can probably establish infection in man as well as some animals and mechanisms by which antibody in conjunction with macrophages can destroy the rickettsiae. However, evidence is accumulating to suggest that T lymphocyte-mediated immunity also plays an important role in the acquired protection against typhus rickettsiae (8-10, 19; sections above and below). The first section above describes unique mechanisms which differ from the classical CMI mechanism of activated macrophages and which are operative on "somatic" and "target cells" as well as on macrophages. However, it is conceivable that non-specifically activated macrophages might also contribute to the control of rickettsiae in tissues. Accordingly, this matter was subjected to critical study in the well-studied and characterized mouse-BCG, C. parvum and Listeria monocytogenes systems, which have permitted others to elucidate so many principles of CMI. We employed Balb/c mice and R. mooseri since this organism establishes a good infection in mice whereas R. prowazekii does not. This system permitted observations on the capacity of activated macrophages to control the rickettsiae (1) without the interfering action of antibody and (2) under the in vivo conditions of the infectious process, as opposed to the artificial conditions in vitro. The latter approach, however, will probably be required to define the intrinsic capacity of activated macrophages to restrict rickettsial growth, since the in vivo system, though it does reproduce the conditions of rickettsial infection (except with respect to antibody), does not ensure that the rickettsiae,

FIGURE 10

RETENTION AND PROGRESSIVE DEGRADATION OF ANTIBODY TREATED VIABLE VIRULENT R. prowazekii (Breinl) within Phagolysosomes in Human Monocyte-Derived Macrophages in Culture.

A. Rickettsia within phagosome shortly after phagocytosis. Note secondary lysosomes heavily labeled with Thorotrast in vicinity, without evidence of fusion yet.

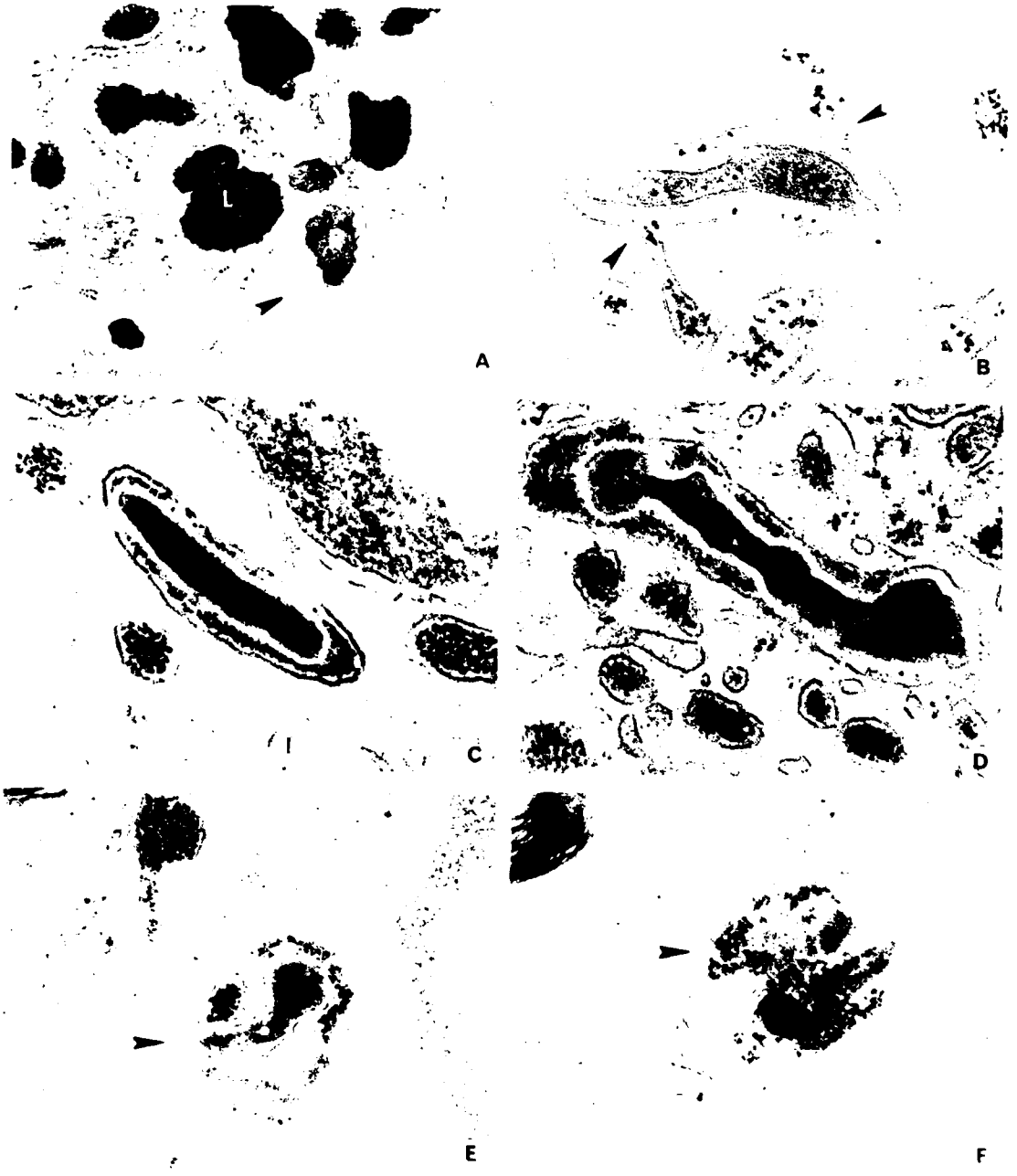
B. Rapid fusion of lysosomes with rickettsia-containing phagosome. Note shrinkage of rickettsia. About 0 min.

C. Phagolysosome containing relatively intact but somewhat shrunken rickettsia and abundant Thorotrast particles from lysosomal fusion. About 10 min.

D. Marked morphological alteration of rickettsia in phagolysosome. About 60 min.

E and F. Advanced morphological degradation of rickettsiae in phagolysosomes. 6 h.

FIGURE 10



multiplying in other target cells, are accessible to the macrophages. Thus the system will more likely yield information on what activated macrophages actually do rather than what they are intrinsically capable of doing if artificially fed rickettsiae. The results of this study, recorded in detail in the accompanying figures, are summarized in outline form below.

1. Development of enhanced levels of non-specific resistance during R. mooseri infection. When mice were infected intravenously with 10^4 PFU of R. mooseri, rickettsial replication in the spleen proceeded rapidly for the first 3 days and then leveled off. After day 5, the rickettsial content of the spleen declined rapidly, reaching relatively low levels by day 7 (See Figure 11). During the period when control of rickettsial replication and clearance was being expressed, there was a markedly enhanced capacity in the spleen and liver to control non-specifically Listeria monocytogenes, an organism extensively used by others to study macrophage activation (Figure 11). In another experiment (See Figure 12), evidence is presented that R. mooseri infection resistance to intravenous infection with different species of facultative intracellular bacteria - viz., S. enteritidis and Y. enterocolitica as well as L. monocytogenes. A third experiment (See Figure 13) showed that the enhanced resistance to L. monocytogenes seen on day 5 of the R. mooseri infection is present at the time of Listeria inoculation and is probably expressed by a population of macrophages with enhanced microbicidal activity, since L. monocytogenes selectively infects macrophages.

Thus, R. mooseri infection of Balb/c mice, either by the i.v. route or s.c. route (data not shown), causes transient increase in non-specific resistance to several facultative intracellular bacteria which coincides with the clearance of R. mooseri from the spleen and which is probably due to the enhanced microbicidal activity of activated macrophages.

2. Failure of non-specific activation of macrophage microbicidal activity to control R. mooseri infection in mouse spleen. When mouse spleen macrophages were activated by i.v. inoculation of BCG or C. parvum according to established procedures to exhibit enhanced non-specific microbicidal activity, as evidenced by enhanced protection against L. monocytogenes, there was no accompanying enhanced capacity to control R. mooseri (See Figure 14). The enhanced capacity to control L. monocytogenes was present at the beginning and end of the R. mooseri assay method (Figure 15) and was not altered by the R. mooseri infection (Figure 16). Thus, splenic macrophages activated by established procedures to show markedly enhanced non-specific microbicidal activity against facultative intracellular bacteria do not appear to contribute significantly to the control of R. mooseri infection in the spleen.

3. Failure of ablation of macrophage activity to enhance R. mooseri replication in mouse spleen. Intravenous injection of silica in amounts sufficient to depress markedly the resistance of mice to L. monocytogenes failed to have any effect on the growth of R. mooseri in mouse spleen (See Figure 17).

FIGURE 11

Development of enhanced levels of non-specific resistance during R. mooseri infection. The 24 hour growth of a standard 10^5 CFU challenge of L. monocytogenes delivered either 1, 3, 5, 7, or 10 days after R. mooseri infection was compared to the growth of the same inocula delivered to control mice. The figure shows that a marked reduction in the numbers of L. monocytogenes were recovered from both livers and spleens of R. mooseri infected mice at a time when rickettsiae were being controlled and eliminated in tissues. The solid line represents the course of R. mooseri infection in spleen following intravenous inoculation. There were 5 mice per group per time point and those groups of animals which showed titers of L. monocytogenes which differed significantly from controls ($P \leq 0.01$) are indicated with an asterisk (*).

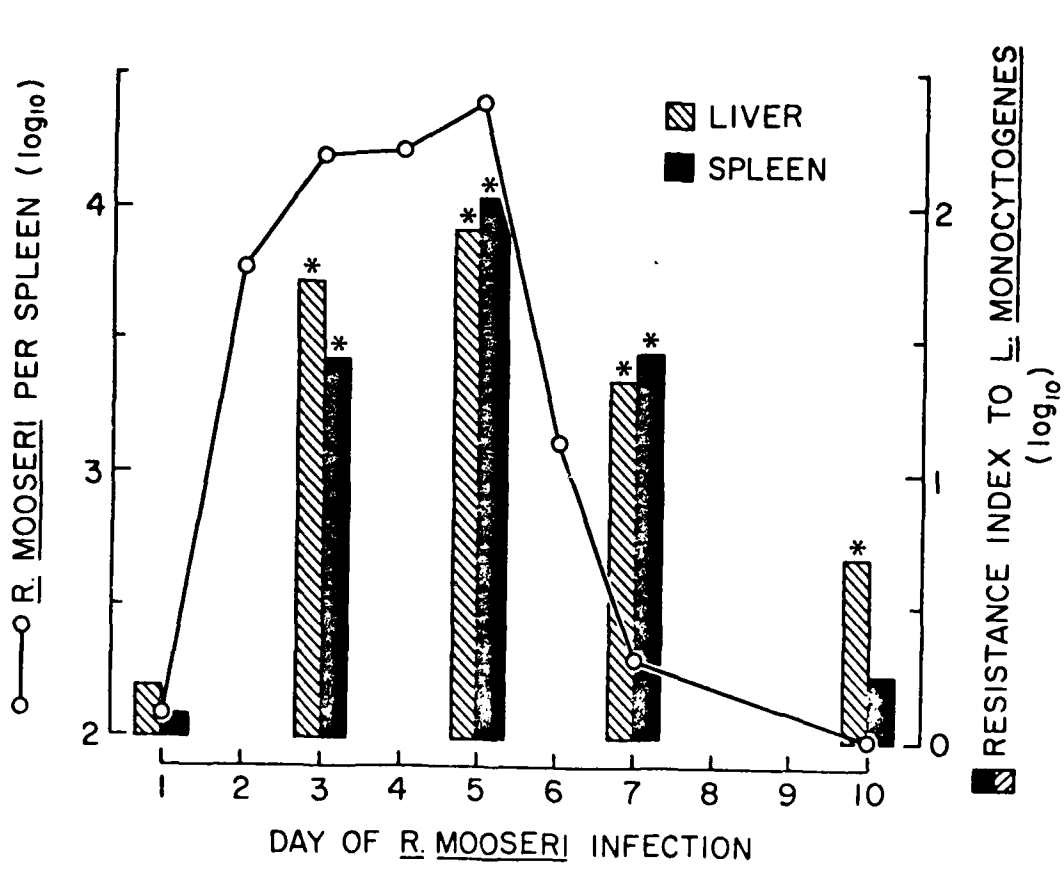


FIGURE 12

Evidence that R. mooseri infection enhances resistance to intravenous infection with different species of facultative intracellular bacteria. Mice were inoculated intravenously with 10^4 PFU R. mooseri and 5 days thereafter separately challenged by intravenous injection of 10^5 CFU of S. enteritidis, Y. enterocolitica or L. monocytogenes. Significantly ($P \leq 0.01$ indicated by *) fewer number of bacteria were recovered at 24 h after bacterial challenge from livers of mice with concomitant rickettsial infection than from livers of controls challenged with the same bacterial inocula. Five mice were used per point.

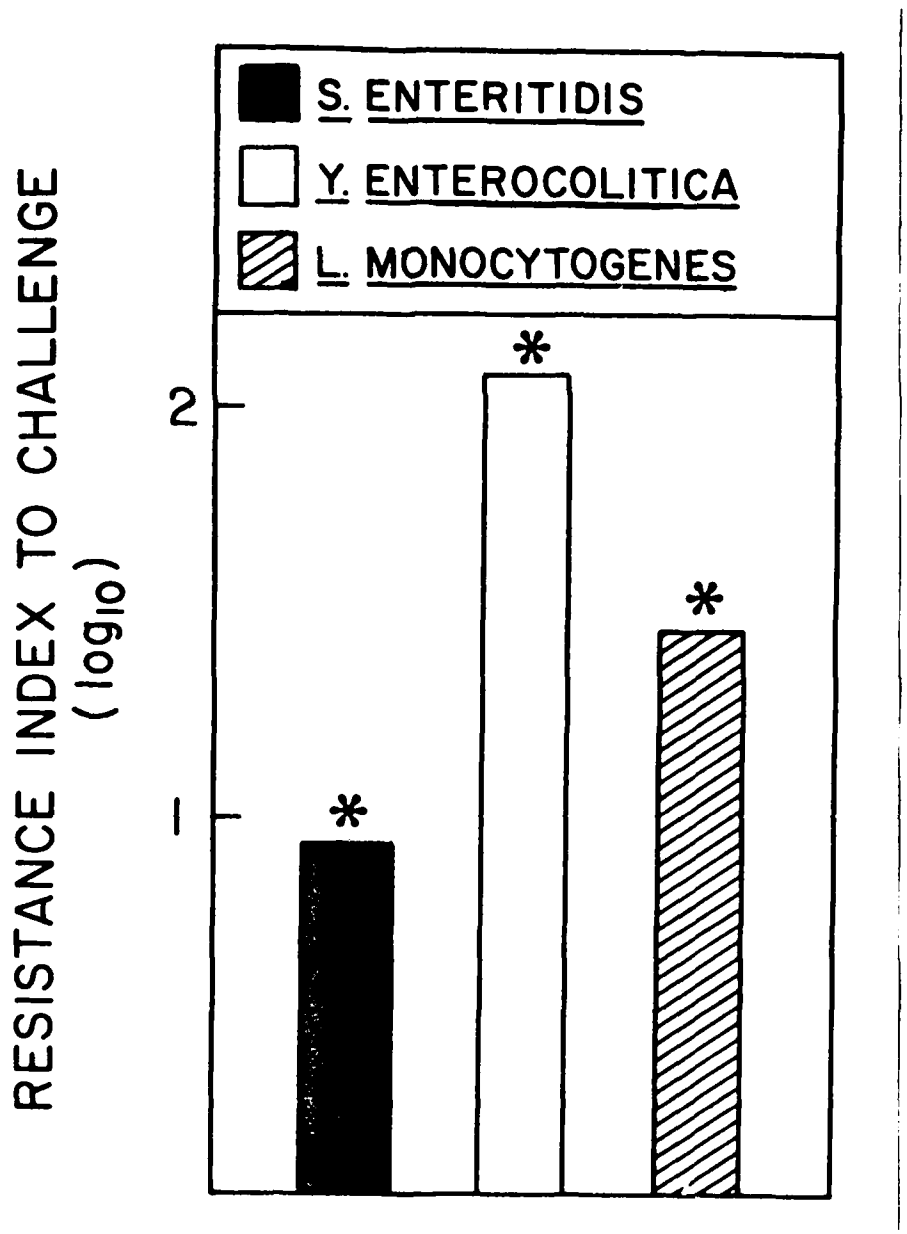


FIGURE 13

Evidence that the enhanced resistance to L. monocytogenes seen at day 5 of R. mooseri infection of BALB/c is expressed by a population of macrophages with enhanced microbicidal capacity. The rapid destruction of Listeria within 12 h in R. mooseri infected mice can only be explained in terms of the enhanced activity of macrophages already present in the liver and spleen at the time of challenge. Five mice were employed per group per time point and those groups of R. mooseri infected mice ( ) which showed significantly ($P \leq 0.01$) fewer bacteria per liver or spleen than controls ( ) are indicated by open symbols.

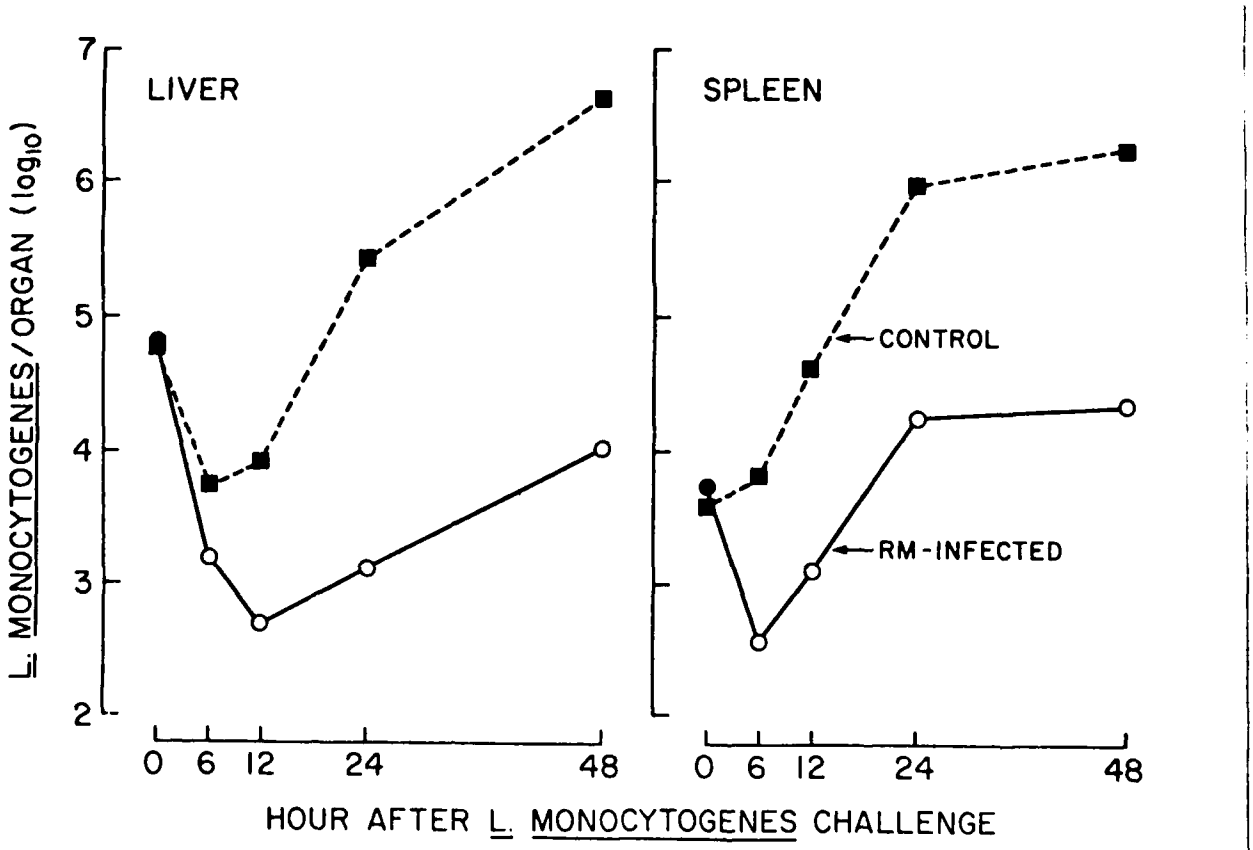


FIGURE 14

Demonstration that nonspecific activation of macrophage microbicidal capacities does not necessarily lead to enhanced killing of R. mooseri. Mice were inoculated with 400 µg of dead Corynebacterium parvum (CP) or 10^7 CFU of live Mycobacterium bovis (BCG). One hour (0 days) or 14 days later subsets of these mice and normal mice were challenged separately with either 10^5 CFU L. monocytogenes or 10^4 PFU R. mooseri. Two days after L. monocytogenes challenge or 4 days after R. mooseri challenge groups of control and treated mice (CP or BCG) were sacrificed and the numbers of listeriae or rickettsiae recovered from spleens determined. Frames A and B show at day 4 after R. mooseri infection that the number of rickettsiae recovered from spleens was similar for control and CP or BCG treated mice. In contrast, significantly ($P \leq 0.01$ indicated by *) fewer numbers of L. monocytogenes were recovered from the spleens of CP or BCG treated mice than from the spleens of controls (frames C & D), an expected result previously described by others ( ). Five mice were used per group per time point.

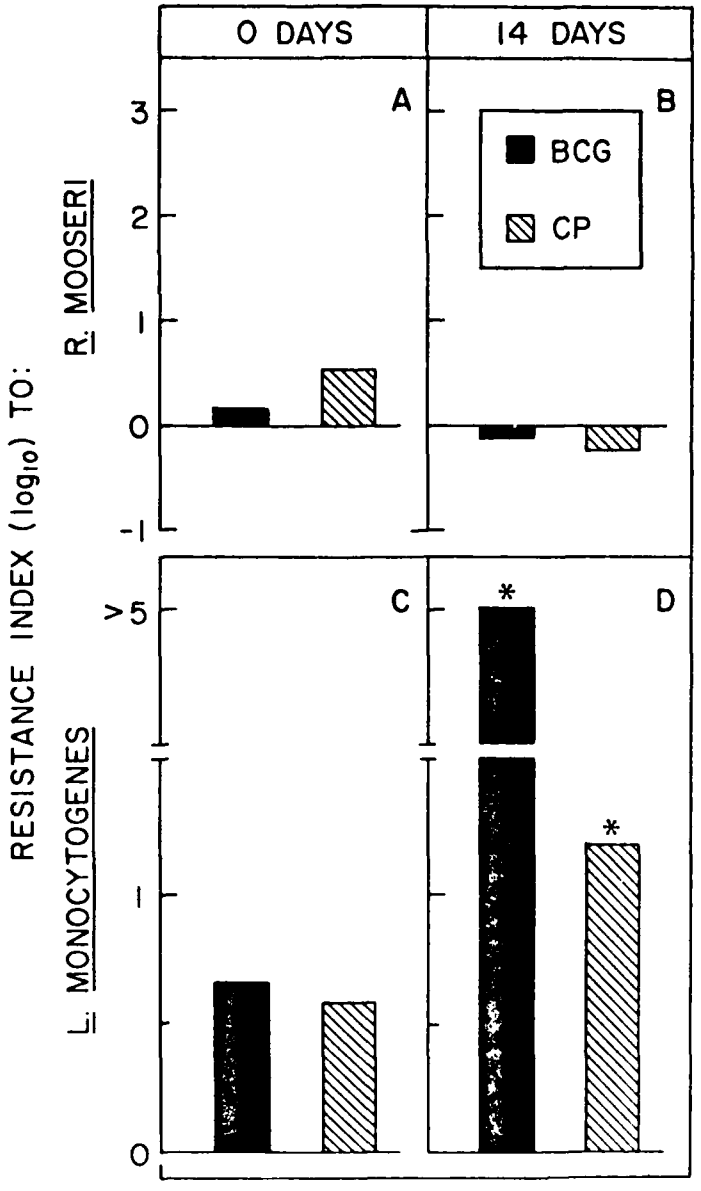


FIGURE 15

Evidence that an established state of macrophage microbicidal capacities exist in BCG infected mice on day 14 through 18 of BCG infection. Mice were inoculated intravenously with 10^7 CFU of BCG and on day 14 or 18 after infection these and normal mice were challenged intravenously with 10^5 CFU L. monocytogenes. Two days after the respective challenges BCG infected and control mice were sacrificed and the number of L. monocytogenes per spleen determined. The figure shows that BCG-infected mice had an enhanced capacity to kill L. monocytogenes on day 14 and 18 of BCG infection as compared to normal mice challenged with the same listerial inoculum. There were 5 mice per group and those groups differing significantly ($P \leq 0.01$) from controls are indicated by an asterisk (*).

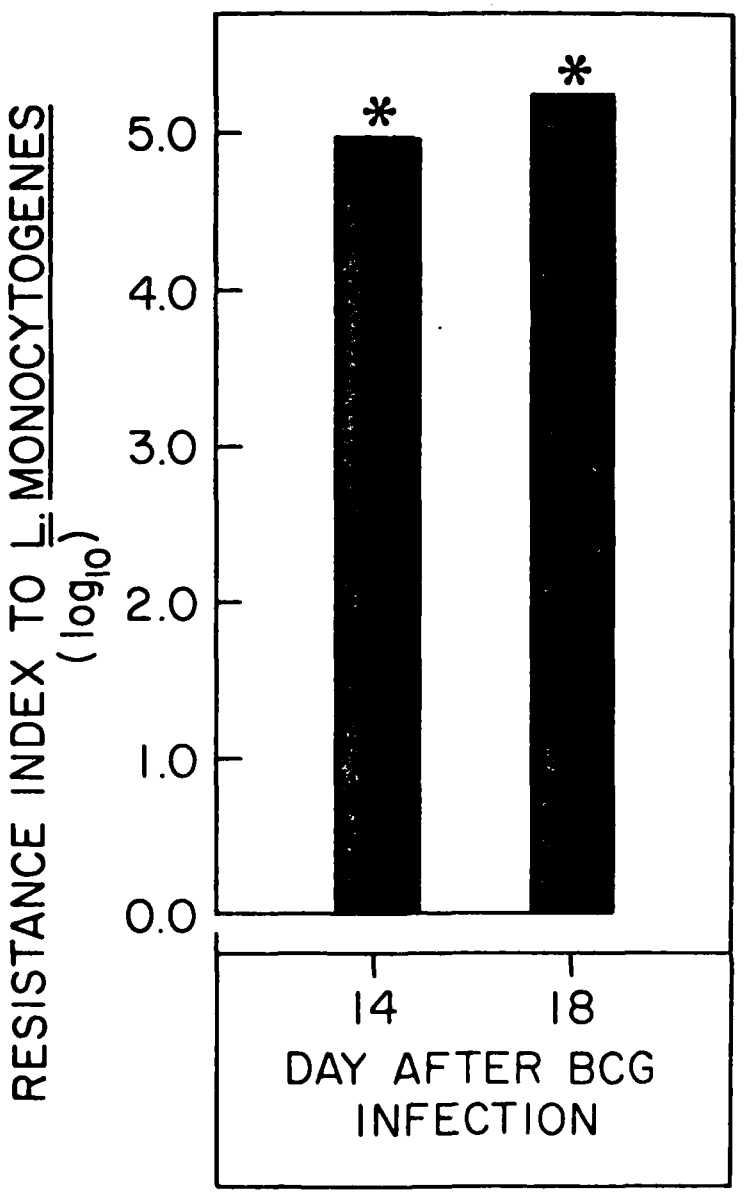


FIGURE 16

Evidence that infection with R. mooseri does not subvert an established state of macrophage microbicidal capacities. Mice were inoculated intravenously with 10^7 CFU of BCG and 14 days later, when enhanced levels of macrophage bactericidal activity had been generated, these mice were inoculated with 10^4 PFU R. mooseri. One hour (0 days) or 4 days after R. mooseri infection of BCG treated mice, these and normal mice were challenged intravenously with 10^5 CFU L. monocytogenes. Frame B of the figure shows that BCG-treated, R. mooseri infected mice maintained through day 4 of the rickettsial infection (day 18 of BCG infection) enhanced levels of macrophage microbicidal capacities. Frame A shows that there was a slight but significant reduction in the number of rickettsiae recovered from BCG infected mice as compared to control mice infected with rickettsiae alone. There were 5 mice per group and those which differed significantly ($P \leq 0.01$) from controls are indicated by an asterisk (*).

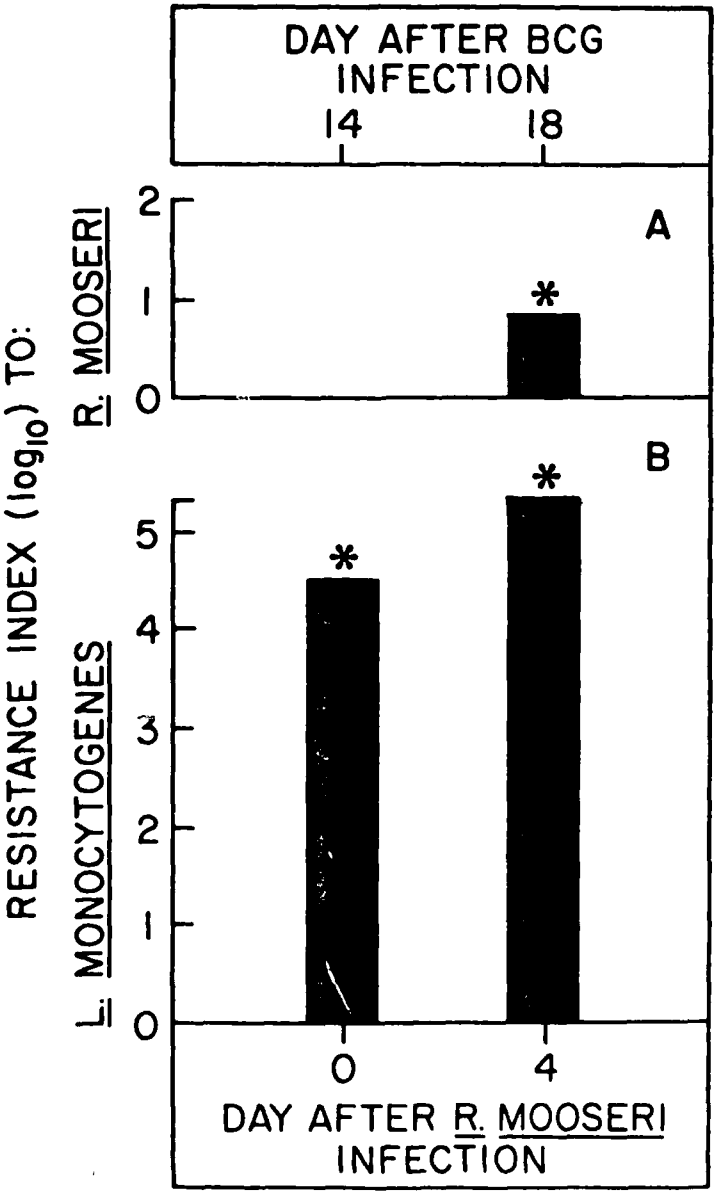
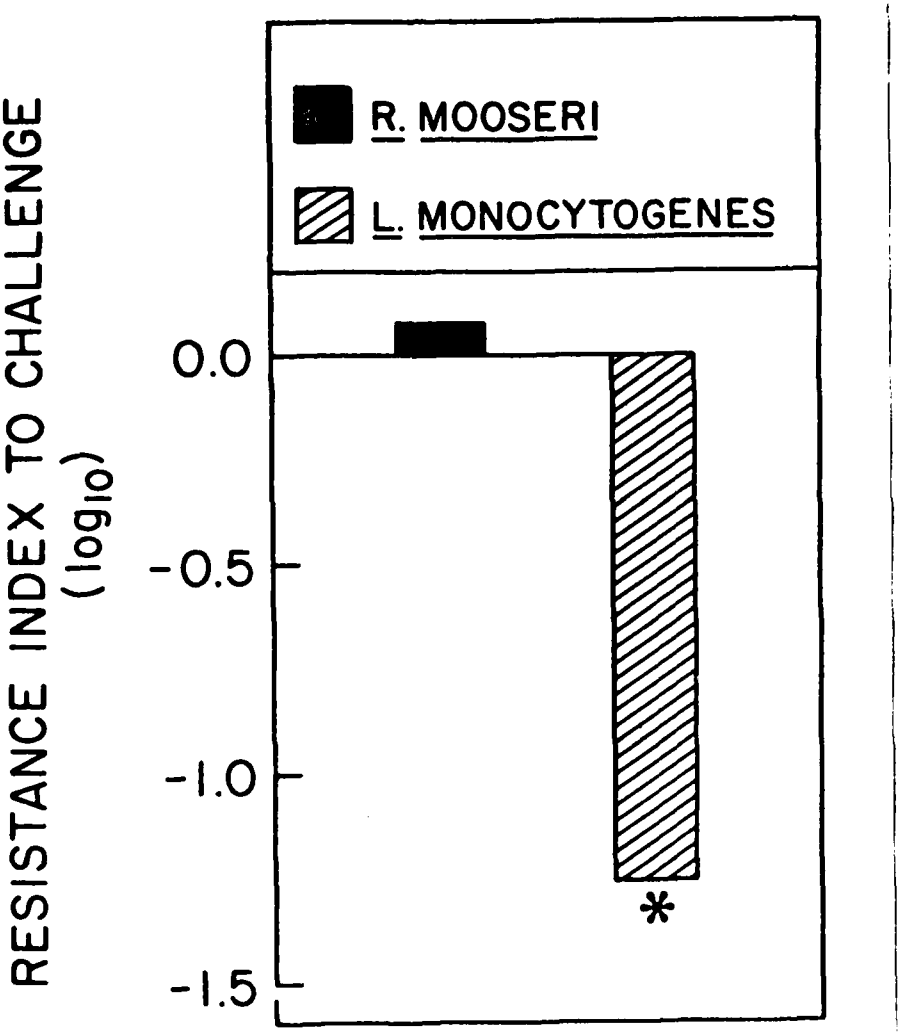


FIGURE 17

Demonstration that ablation of macrophage microbicidal capacities does not result in an uncontrollable and rapidly progressing R. mooseri infection. Mice were inoculated intravenously with 3 mg of silica and 24 h later subsets of these mice and normal mice were challenged separately with either 10^5 CFU L. monocytogenes or 10^4 PFU R. mooseri. One day (L. monocytogenes) or 4 days (R. mooseri) after the respective challenges groups of control and treated (silica) mice were sacrificed and the number of listeriae or rickettsiae recovered from spleens determined. It can be seen that significantly ($P \leq 0.01$ indicated by *) greater numbers of listeriae but not rickettsiae were recovered from spleens of silica treated animals. Five mice were used per group.



Thus, in the R. mooseri - mouse model, R. mooseri infection elicits the production of activated macrophages with enhanced non-specific microbicidal activity against facultative intracellular bacteria, the classical effector mechanism for expression of cell mediated immunity in tuberculosis or listeriosis. Yet, considerable evidence is presented to suggest that such activated macrophages alone do not participate to any great extent in the observed control and clearance of R. mooseri during infection. Since T-lymphocyte mediated mechanisms appear to be required for the control of typhus rickettsiae in tissues, these results suggest that some mode of expression of CMI is operative other than the classical non-specifically activated macrophage and enhance the potential significance of the mediators described in the first section which act upon infected "somatic" or "target" cells. This does not mean, however, that macrophages in conjunction with specific antibody do not perform important clearance functions.

D. Dependence of CMI to R. mooseri infection in mice on thymus-derived lymphocytes from immune animals. (With A. Crist). Early studies in these laboratories suggested a correlation between the development of immunity following vaccination with the living attenuated E strain of R. prowazekii and the appearance of delayed type hypersensitivity detected by skin tests (19). Our more recent experiments with R. mooseri infection in guinea pigs showed that recovery from infection was accompanied by the development of the capacity of blood lymphocytes to produce MIF upon stimulation with R. mooseri antigen (Gluck, L., R.W.I. Kessel and C.L. Wisseman,Jr., to be published). Moreover, lymphocytes from persons convalescent from typhus infection were shown to undergo lymphoblast transformation upon stimulation with R. prowazekii antigen (L. Fiset and C.L. Wisseman, Jr., to be published). Finally, again in the R. mooseri-guinea pig model, it was shown that R. mooseri replication at the site of inoculation in the skin could be controlled by the adoptive transfer of spleen cells from immune animals but not by the transfer of immune serum (8-10). However, both transferred immune spleen cells and immune serum could alter the manifestations of systemic infection (Murphy, J.R., C.L. Wisseman, Jr. and P. Fiset, in press). Though indirect evidence suggested that immune T-lymphocytes were probably responsible for effecting control of rickettsial replication within tissue sites of infection, as skin, formal proof that this was the case was not accomplished with the guinea pig system.

Since convenient methods have been devised for preparing lymphocyte suspensions enriched in either B or T lymphocytes from mice and since inbred mouse strains are readily available, an experimental system employing R. mooseri infection in Balb/c mice was developed to perform the formal identification of cell type responsible for controlling rickettsial proliferation in tissues, to confirm the observations on immune serum and to permit further detailed immunobiological studies. For the present studies, mice which were to serve as donors of serum or cells were infected by inoculating 10^4 PFU R. mooseri into the left hind footpad. The assay for the control of rickettsial proliferation consisted

of inoculating non-immune mice intravenously with 10^4 PFU R. mooseri, administering serum or cells 6 h later (after the rickettsiae had established tissue infection so as to immunize the effects of differential clearance) and then determining by plaque count the number of R. mooseri in spleen homogenates at intervals thereafter. The growth pattern with respect to time was compared in control and treated animals. The main results are briefly outlined in narrative form below. A publication describing these studies in detail is in an intermediate stage of preparation, but the figures have not been drawn for publication.

As in the case of guinea pig skin, passively transferred immune serum did not influence the growth of R. mooseri in mouse spleen. No further studies were done with immune serum.

However, after a lag of about 2 days, control of spleen infection was evident in animals which had received immune spleen cells by the intraperitoneal route.

Certain properties of the cell system were systematically investigated. Thus, the capacity of donor spleen cells to confer the capacity to control R. mooseri infection in the spleen of non-immune mice was barely detectable 7 days after donor infection but was pronounced at 14, 21 and 28 days, the last time point tested. The capacity of immune spleen cells to inhibit R. mooseri infection in recipient animal spleen was expressed more rapidly and more strongly when the cells were administered intravenously than when administered intraperitoneally. Protection conferred by immune spleen cells was pronounced when the number of cells was 10^8, less pronounced with 10^7 cells and not measurable by the methods employed with 10^6 or fewer cells.

The T-lymphocyte was identified as the cell in the spleen homogenates which conferred protection. Thus, treatment of immune cell suspensions with anti-mouse immunoglobulin antiserum plus complement did not diminish the capacity of the suspensions to confer protection whereas treatment with anti-theta antiserum markedly diminished or ablated the capacity to confer protection.

Some other properties of the cell system were also investigated. These suggest, but do not yet prove, that at least two kinds of cell are involved: (1) a recirculating memory type cell, effective by either intraperitoneal or intravenous route, which after specific stimulation undergoes replication prior to development of (2) a blastoid cell which does not recirculate, enters (homes) but does not necessarily easily leave the area of action. Thus, the requirement for viable cells capable of replication for successful transfer of protection was demonstrated in experiments which showed that repeated cycles of freezing and thawing, x-irradiation or mitomycin C treatment of cells or vinblastin treatment of donors ablated the capacity to confer protection.

Immune spleen cells confer protection whether given by the intraperitoneal or intravenous route. However, peritoneal exudate cells from immune mice confer protection when given intravenously but not when given intraperitoneally.

Thus, these studies with the Balb/c mouse-R. mooseri model (1) confirm the inability of immune serum alone to control rickettsial replication locally in an infected tissue or organ and (2) establish the effector cell which is responsible for controlling rickettsial replication at the tissue/cell level as a thymus dependent lymphocyte. Moreover, they suggest that this mouse model, or some modifications thereof, may be useful for elucidating many important details of the immunological mechanisms in R. mooseri infections.

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II. STUDIES ON RICKETTSIAL NUCLEIC ACIDS, PROTEINS AND SELECTED FUNCTIONS

A. Studies on the genome of rickettsiae. As a part of studies to establish the basis for genetic investigation of rickettsiae as well as to aid in the classification of rickettsiae, especially of the spotted fever group which is in terrible disarray with apparent new organisms being isolated around the world with increasing frequency (including our series of serologically distinct strains from Pakistan), we initiated studies on rickettsial DNA which included the determination for different groups and species of genome size, % G+C and degree of hybridization. Some of these studies were reported in the previous annual report and have just been published or are actually in press. Thus, one study, which compared in detail strains classified on conventional grounds as either R. prowazekii or R. mooseri, showed that (1) all strains had a similar % G+C content and a similar genome size (about 1.1×10^9 daltons), (2) the DNA of strains that had been classified as R. prowazekii or R. mooseri hybridized to nearly 100% with each other but (3) the DNA of R. prowazekii strains hybridized with that of R. mooseri strains to only 70-77% (18). Another study (17) showed, among other things, that validated strains of Rochalimaea quintana, whether of old world or new world origin, hybridized to nearly 100% with each other but hybridized to a very low degree with Baker's vole agent (1), establishing it as probably a separate species of the genus Rochalimaea. Other investigators had proposed, on the basis of certain biological and biochemical properties, that Baker's vole agent was simply a strain of R. quintana (14,27). This, of course, opens the possibility that there may be other species of *this genus in nature* where the basic adaptation is the ecological niche of pericellular parasitism.

We have recently been able to plaque purify Rickettsia canada, whose available yolk sac seeds contained an extraneous "egg" bacterium difficult to circumvent and have, thus, been able to conduct similar studies on it. The results are presented below.

Up to this point we have used optical methods (8,10,13) to determine DNA melting points for % G+C determinations and reassociation kinetics for determination of genome size and degree of hybridization. These methods require relatively large amounts of DNA, which are feasible with typhus group rickettsiae and Rochalimaea species but, pose serious limitations with spotted fever group because yields of organisms are substantially less. Therefore, more sensitive methods using the principles of intense radiolabelling of DNA by nick-translation methods (11,20) and determination of free reassociation kinetics for genome size and degree of hybridization (6,7,9,24) are being adapted (see "c" below).

a. Genome size of R. canada and degree of DNA hybridization with typhus and spotted fever group rickettsiae (Myers, Smith, Oaks).

A third species, Rickettsia canada, was added to the typhus group of rickettsiae, following its isolation by McKiel, Bell and Lackman in 1963 (12). This new agent was isolated from Haemaphysalis leporispalustris ticks removed from rabbits near Richmond, Ontario. It was found to possess complement fixation antigens in common with R. prowazekii and R. mooseri as

shown by cross reactions with antisera prepared in guinea pigs, rabbits and hamsters. However, tests with mouse antisera suggested that it was different from the two known species, R. prowazekii and R. mooseri. In 1968, acute and early convalescent serum specimens from a patient suspected of having Rocky Mountain spotted fever were submitted to WRAIR for confirmatory serology. Complement-fixation tests with soluble antigens prepared from the spotted fever group failed to demonstrate antibodies, but tests with group and specific antigens from the typhus group established a relationship with R. canada. As a result of this finding, a follow-up study was done on some 70 sera where RMSF was diagnosed clinically; 10 of these could not be confirmed earlier by serology (3). The new serological data developed indicated the possibility that 4 of these patients may have experienced a severe febrile illness due to R. canada. In this new study positive complement-fixation titers were not found when the sera from these 4 patients were reacted with specific complement-fixing antigens from R. prowazekii or R. mooseri.

The biological properties of R. canada are in several ways, however, more attuned to the spotted fever group. Burgdorfer and Brinton (1970) (4) showed that when R. canada was grown in several species of ticks, the rickettsiae were noted not only in the cytoplasm but also in the nuclei of infected cells. It was also noted that infection with R. canada could be transmitted transovarially, since larvae were observed to be infected. The ability of rickettsiae to grow in cytoplasm as well as in nuclei of infected cells has not been associated previously with rickettsial species other than those belonging to the spotted fever group. Thus, while R. canada shows some serological relationship to the typhus group, and possesses a G+C ratio in the typhus group (26), its pattern of infection in several species of ticks is strikingly similar to that of spotted fever rickettsiae; it produces generalized infection of all tick tissues, exhibits intranuclear as well as cytoplasmic growth, and appears to be passed via eggs to the progeny.

Studies in progress in these laboratories seem to support the notion that perhaps R. canada is not as closely related to the typhus group as was suggested by earlier serological data. Table 6 presents data on the genome sizes of R. canada and R. rickettsii based on the inital rate of DNA renaturation. It can be seen that the genome sizes for the two organisms are quite similar and are approximately 1.48×10^9 daltons. This value differs significantly from the value 1.1×10^9 daltons, which is the approximate size for both R. prowazekii and R. mooseri (18). It is generally accepted that significant or large differences in genome sizes between two microorganisms preclude their having a close taxonomic relationship.

Table 7 presents data on the degree of DNA-DNA hybridization between R. prowazekii, R. mooseri, R. canada and R. rickettsii. The values for the various DNA combinations were consistently low, varying between approximately 30 to 50 percent. This is in contrast to the nearly 100% hybridization values found when various strains within a species (R. prowazekii or R. mooseri) were hybridized together. When R. prowazekii and R. mooseri DNAs were hybridized the values were consistently in the range of 70 to 77%.

Conventional SDS-polyacrylamide gel electrophoresis patterns of whole organism proteins, stained with Coomassie blue, of R. prowazekii (Breinl), R. canada, R. mooseri (Wilmington) and R. rickettsii (Sheila Smith) (Figure 18) showed that each organism had a distinct and unique pattern. Nevertheless, there were substantial similarities, both general and restricted. Thus, several major and minor proteins from all 4 organisms had similar migration patterns. Some bands of similar migration were found between R. canada and R. prowazekii, R. canada and R. rickettsii, and R. canada, R. mooseri and R. rickettsii. Although similar migration does not necessarily indicate identity, among closely related organisms there is an increased probability that some may be identical, especially when significant degrees of DNA homology exist and serological cross-reactions are known to occur. Although the 30-40 bands visible on this photographic reproduction of a one-dimensional polyacrylamide gel pattern represent only a minor fraction of the total number of peptides theoretically coded for in the rickettsial genome, these patterns nevertheless show that (1) each organism has a unique overall pattern, (2) there are certain general as well as specific similarities in various combinations and (3) there are substantial differences.

Thus R. canada appears to possess some features of both typhus and spotted fever groups, as well as some unique features of its own:

1. In common with the typhus group: shared antigens which cross-react, at least in 1 direction (but the typhus and spotted fever groups also cross-react) (17,18); a similar % G+C.

2. In common with the spotted fever group: biological features (tick-borne; transovarial transmission; intranuclear growth); similar genome size.

3. It differs from both by: relatively low degree of DNA:DNA hybridization; PAGE protein profile (though all 3 have some bands which do appear similar). This can be evaluated much better when similar data are available from other members of the spotted fever group.

On the basis of the DNA and protein studies, the following considerations emerge.

1. The % G+C is only compatible with possible relationship to the typhus group, but does not prove a relationship because DNA's of the same overall nucleotide composition can have very different sequences. It suggests a difference from the spotted fever group, but the difference is relatively small and may not absolutely exclude the possibility of a relationship to the spotted fever group.

2. The genome size suggests a substantial difference from the typhus group. It is compatible with a relationship to the SF group, but does not prove such a relationship.

3. The DNA:DNA hybridization studies establish neither a clear close relationship with one group or another nor differences which exceed those commonly found among different species of the same bacterial genus.

4. The PAGE protein profiles show both substantial differences and some apparent similarities among all 4 organisms tested.

FIGURE 18

Discontinuous polyacrylamide gel electrophoresis patterns for whole cell digests of R. prowazekii (Breinl), R. canada, R. mooseri (Wilmington), and R. rickettsii (Sheila Smith) stained with Coomassie blue.

Note: Although certain bands appear to be common to two or more organisms, each organism appears to have a distinctly different overall pattern.

FIGURE 18

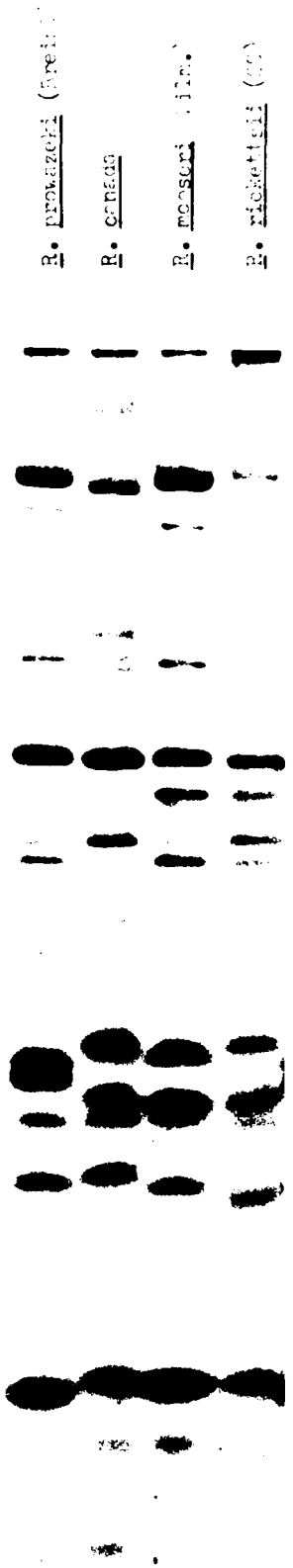

Table 6. Genome size of R. canada and R. rickettsii based on initial rate of DNA:DNA renaturations.

Organism	Genome size Mol. wt. X 10^{-9}	Mean ± S.D.
R. canada - Lot A	1.53	1.48
	1.53	
	1.44	n = 9
	1.53	$\bar{x}$ - 1.48
	1.41	Sx - 0.04
R. canada - Lot B	1.50	$S\bar{x}$ - 0.01
	1.47	
	1.47	
	1.47	
R. rickettsii (Sheila Smith)	1.56	1.48
	1.35	n = 7
	1.60	x - 1.48
	1.40	Sx = 0.12
	1.46	$S\bar{x}$ - 0.04
	1.63	
	1.35	

Table 7 DNA-DNA hybridization values between R. prowazekii, R. mooseri, R. canada and R. rickettsii as measured by initial rate of DNA renaturation

Hybridization pairs	% Hybridization			
	D_1[1]		D_s[2]	
	Exp.	Av.	Exp.	Av.
UNEQUAL SIZES				
R. prowazekii (Breinl) vs. R. rickettsii	38 42 35	38	47 53 60	53
R. mooseri (Wilm.) vs. R. rickettsii	28 26	27	36 35	35
R. canada vs. R. prowazekii (Breinl)	35 35 40	37	42 43 47	44
EQUAL SIZES				
R. canada vs. R. rickettsii	51 51			

1. D_l = degree of hybridization to large gonome

2. D_s = degree of hybridization to small genome (see footnote).

Footnote to Table 7

When DNA:DNA hybridizations are conducted between two organisms whose genomes differ appreciably in size, it is not possible to express the degree of hybridization as a single value. The formulas for determining the degree of hybridization for unequal size genomes are: $D_L = \frac{4\ V_m - (V_L + V_S)}{2\ V_S}$ and $D_S = \frac{4\ V_m - (V_L + V_S)}{2\ V_L}$, where D_L is degree of hybridization to the large genome, D_S is degree of hybridization to the small genome, V_m is renaturation rate for a 1:1 mixture of the two DNAs, V_L is renaturation rate of the large genome DNA on itself, and V_S is renaturation rate of the small genome on itself. In the case of equal sized genomes the formula is: $D = \frac{4\ V_m - (V_A + V_B)}{2\sqrt{V_A V_B}}$ where V_A and V_B represent the rate of renaturation for the two different DNAs involved.

At this point in our evolving knowledge of the relationships between organisms currently classified in the genus Rickettsia, our best estimate of the position of R. canada is that it likely represents a new and separate biogroup comparable in status to the typhus and spotted fever biogroups.

b. Determination of DNA base ratio and genome size in Coxiella burneti. We completed recently a study of the deoxyribonucleic acid of Coxiella burneti. A frozen lysate of purified C. burneti was obtained from Dr. Oswald Baca, University of New Mexico, Albuquerque. The DNA was purified by a combination of enzyme digestions, phenol extraction, and hydroxyapatite adsorption and elution. The purification method is fully described in a recent publication (18). The purified DNA was sheared as two separate lots (A and B) in the Ribi cell fractionator to a molecular weight of approximately 2.5×10^5. Lots A and B were stored in 0.5 ml portions at $-70^{\circ}C$ for further study.

The DNA base ratio was determined by the melting point method of Marmur and Doty (13) with some modifications (18). The percentage of guanine plus cytosine was determined in two separate experiments to be 42.9% and 42.5%. These values compare well to those values (42-44%) which have been described by others (21,22,23).

The genome size determination was determined by the initial rate of DNA renaturation (10). We have applied this method to the genome size of the typhus group, which is described in detail in our recent publication (18). Table 8 shows the values obtained for lots A and B. The DNA was sheared as two separate lots as a sort of internal control on the procedure, since the values obtained are a function to a slight extent of DNA fragment length. It can be seen that the mean values for lots A and B are nearly identical and are approximately 1×10^9 daltons. This is very close to the values that we obtained previously for the typhus group as well as Rochalimaea quintana. Schramek (21) described the isolation and characterization of DNA from C. burneti. The DNA was subjected to buoyant density gradient centrifugation in cesium chloride. An analysis of the sedimentation pattern indicated a rather large distribution of particle size for the final isolation product. Its average molecular weight was 1.8×10^7 daltons. Although Schramek had taken care to minimize shear of the DNA in his procedure some undoubtedly occurred. Schramek made no claim that the fragment length obtained necessarily represented that of the intact genome. In contrast, the procedure that we employed requires that the DNA be sheared to a certain size by employing a French pressure cell.

Although its genome size is nearly the same as that of the typhus group, the fact that there is an 11-13% difference in its guanine plus cytosine content as compared to the typhus and spotted fever groups makes it taxonomic relationship to the latter groups quite remote.

c. Adaptation of nick-translation radiolabeling of rickettsial DNA and free reassociation kinetics for determination of genome size and degree of hybridization. We have succeeded in preparing yolk sac suspensions of the 20 spotted fever group rickettsiae (established species and isolates from Pakistan, Israel, Thailand and Czechoslovakia). The yields are small compared

Table 8. Genome size of C. burneti based on initial rate of DNA:DNA renaturation

Organism	Genome Size Mol. wt. x 10^{-9}	Mean ± S.D.
C. burneti - Lot A	1.07 1.00 1.04 1.10 1.00	1.04 ± 0.04
C. burneti - Lot B	1.11 0.98 1.08 1.02 1.02	n = 10

with those from the typhus group. Although with great effort we have been able to produce enough R. rickettsii to provide DNA for % G+C and genome size measurements and preliminary DNA:DNA hybridization studies with R. prowazekii, R. mooseri and R. canada by our established optical methods (as in "a" and "b" above), it became obvious that we could not prepare similar quantities of DNA from all of the spotted fever group strains which we wished to compare by hybridization within a reasonable time or at a reasonable cost (materials and labor). A more sensitive method was required. Although theoretically the optical method could be scaled down by a factor of 5-10, it is likely that major technical problems would be encountered. Moreover, conventional isotope-membrane techniques require amounts of "cold" DNA which approaches that required by our current optical methods. Hence, we investigated alternative methods. The most likely method found which might yield the desired results with amounts of DNA an order of magnitude or less below those currently required by the optical methods involved the production of DNA with an extremely high specific activity by the nick-translation method, reassociation with "cold" DNA in free solution, and trimming the loose ends and unassociated loops with S enzyme.

It is our intention to employ radiolabeled rickettsial DNA to carry out studies on genetic relatedness within the spotted fever group. It will be necessary to prepare radioactive DNA at a high level of specific activity in order to obtain the required degree of sensitivity, and thus conserve rickettsial DNA. In the past, viral or bacterial DNA was labeled primarily by in vivo techniques (9,24). This approach to labeling is not very appropriate for intracellular parasites, such as rickettsiae, since the labeled DNA precursor is once removed from the organism by the competing host cell. It is doubtful whether the specific radioactivity obtained in the rickettsial DNA would be adequate for these studies.

Over the past several years, laboratories have begun using in vitro procedures to label DNA to higher specific radioactivity than that which can be achieved by in vivo labeling. One procedure for in vitro labeling is based on the ability of E. coli DNA polymerase I to catalyze nick-translation (11,20). A number of viral DNAs have been so labeled; e.g., SV 40, lambda phage, polyoma, and adenovirus 2 (20), and specific activities greater than 10^8 cmp/µg DNA have been achieved. This has greatly improved the sensitivity of nucleic acid hybridization technology.

The method for radiolabeling DNA using the nick-translation reaction is based on a study by Kelley et al. (11) in 1970 describing replication of DNA by E. coli DNA polymerase I. These investigators reported that before chain copying can occur using purified polymerase I, scissions or nicks have to be introduced into a single strand. Pancreatic DNA ase I is readily used to accomplish nicking in vitro. DNA polymerase I requires a terminal nucleotide bearing a 3' hydroxyl group from which the DNA chain is extended. DNA polymerase I has both exonuclease and polymerase activity. Thus, as nucleotides are removed and added simultaneously, the nick is moved or translated linearly along the DNA chain without net DNA synthesis taking place, the process being

referred to as nick-translation. In the presence of labeled deoxyribonucleoside triphosphates, preexisting unlabeled nucleotides in the DNA chain are replaced by radioactive duplicates. Specific activity of the DNA product depends on the specific activity of the labeled nucleotide and the extent of nucleotide replacement.

The protocol for the nick-translation labeling of rickettsial DNA follows. Radiolabeled deoxy TTP will be employed as the radioactive tracer, either ^{3}H or ^{32}P labeled. A somewhat higher specific activity is obtainable with ^{32}P, but, of course its radioactive half-life is only 14 days compared to 12 years for ^{3}H. Approximately 50 µCi of tracer are added to the reaction tube (sp. ac. of >300 Ci/m mol). The tracer is lyophilized in a vacuum desiccator. The following reagents are then added:

Nick-translation buffer	5 µl
Cold deoxynucleoside triphosphate mixture	4 µl
0.5 g rickettsial DNA	2-7 µl (depending on concentration)
Translation grade water	0-5 µl (according to DNA volume)
DNA polymerase I	2 µl
DNA ase I	2 µl
Total volume	20 µl

The tubes are capped and incubated at 12 to 14°C in a bath. For a kinetic study of the labeling procedure, 1 or 2 µl aliquots are withdrawn at appropriate time intervals (0 - 2 hrs.) and added to tubes containing translation stop buffer (sodium dodecylsulfate and a large excess of cold (non-radioactive DNA). Following addition of 2 ml of cold 10% trichloroacetic acid, the precipitated DNA is collected on filter discs using a vacuum filtration apparatus. Filters are washed with 10% TCA, dried thoroughly, and the radioactivity determined by liquid scintillation counting. This procedure will allow us to determine the conditions for obtaining maximal DNA radioactivity. When the DNA is intended for further DNA hybridization studies, the enzymatic reaction will be stopped by other means, either the addition of a proteolytic enzyme (proteinase K or trypsin) to enzymatically degrade the polymerase and DNAase, or by extraction of the reaction mixture with phenol to separate protein from DNA.

In our projected studies on genetic relatedness within the spotted fever groups we will continue to use free-solution reassociation techniques to measure the degree of DNA:DNA hybridization. Free solution is preferable to those techniques where one of the DNAs is immobilized, such as on a membrane surface. Problems are encountered in the latter technique due to a certain amount of leaching of DNA from the membrane during the hybridization period. In addition, the degree of binding of labeled DNA fragments to unlabeled DNA from the same source is 10 to 70% on filters, and from 75 to 95% in free solution. Finally, kinetics in free solution are typically uncomplicated second-order, whereas kinetics on filters are more complex.

In our previous study on the genetic relatedness of the typhus group, DNA:DNA hybridization occurred in free-solution. The two DNAs were present in equal concentrations and neither was radiolabeled. The progress of DNA annealing was monitored optically by measuring the decrease in absorbance at 260 nm. This proved a very useful technique. However, about 10 µg of each DNA was required. In our new studies on the spotted fever group, free-solution reassociation will still be employed, but one of the DNAs will be radiolabeled by the in vitro nick translation technique previously described. The kinetics of free-solution reassociation is now well understood and it is possible to adjust the concentrations of the radiolabeled DNA and the unlabeled DNA such that at most 1-2% of the radiolabeled DNA anneals on itself while >90% of the unlabeled DNA anneals on itself within a given time period. In practice, this involves using a thousand to ten thousand greater quantity of the unlabeled DNA relative to the labeled component. Since both DNA components represent rickettsial DNA it is important to conserve both. The total amount of unlabeled DNA required will be minimized by (i) producing a labeled DNA with a very high specific activity, (nick-translation method), and (ii) performing the hybridization reaction in the smallest volume possible (ca. 5 to 10 µl). Thus, in a typical hybridization reaction involving homologous labeled and unlabeled DNAs, 0.001 µg of labeled DNA (10^7 dpm/µg) would be reacted at 60°C with 1 µg of unlabeled DNA in a 10 µl volume for the time required for >90% reassociation of the unlabeled DNA (about 16 hrs). The free-ends and open loops of the reassociated DNA would be enzymatically removed by the S, nuclease method. Any non-associated single-stranded DNA would be reduced also to the single nucleotide stage. This enzyme has been shown recently (25) to be highly specific under appropriate conditions in cleaving single stranded DNA to the nucleotide stage while having virtually no effect on double-stranded DNA. (The S nuclease procedure has been applied in a number of DNA hybridization studies in recent years (2, 6, 7, 15, 16)). After this nuclease step a carrier DNA such as salmon sperm DNA, or some other DNA, is added to provide bulk. The DNA is then precipitated with cold trichloroacetic acid and the precipitate is collected on membrane filters. A thorough washing with TCA removes radiolabeled nucleotides. The membrane is then transferred to a scintillation vial and dissolved with methyl cellosolve. The scintillation cocktail is then added and the sample is counted. The counts obtained are then normalized against the homologous reaction and expressed as a percentage of the latter.

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B. Proteins of R. prowazekii. (Oaks, Smith)

Knowledge of the proteins of rickettsiae has many potential uses, among which are the identification of antigens which are involved in the different types of immune response, the production of purified "sub-unit" vaccines, the development of highly specific diagnostic reagents and the clarification of the relationships among rickettsiae and their taxonomy. Moreover, the reasonable possibility that different strains of the same species may show unique protein components might serve as an important marker for epidemiological studies. Accordingly, we are developing simplified, sensitive analytical methods which involve selective rickettsial radiolabeling of proteins in tissue culture, minimal purification, immunoadsorption as desired, one and two-dimensional electrophoresis by SDS-polyacrylamide gel and isoelectric focussing techniques and radiofluorography. Progress along these lines is described below.

1. Growth of radioisotope-labeled rickettsiae in L-cells on microcarrier beads. In order to produce radioactive rickettsiae with a very high specific activity, a system is needed that will produce a high titer of rickettsia in a minimal volume, containing radioactive precursor molecules, such as amino acids. To overcome the disadvantages of conventional systems, microcarrier beads were used to grow and label Rickettsia prowazekii. This system not only produces a large number of rickettsia, but also, the high cell density achieved allows the use of small volumes of medium containing high concentrations of radioactive isotopes. The microcarrier system also simplifies many procedures used in tissue culture and in rickettsiology.

a. Description of microcarrier system. Pharmacia microcarriers at a concentration of 4 grams per liter are seeded with approximately 6×10^4 cells per ml. Cell types used are either L-cells or primary chick embryo cells. Spinner flasks containing the culture are incubated at 37°C and within two days the cells reach a monolayer on each bead. Cell counts are performed on aliquots of the bead culture by using versene or trypsin to remove the cells from the beads, and then counting in a hemocytometer. In our system, approximately $5\text{-}10 \times 10^5$ cells per ml of culture medium are present at monolayer.

The microcarrier cultures are infected by allowing the beads to settle into a reduced volume to which is added approximately 10 rickettsiae per cell. The beads are slowly spun at 34°C for 60 minutes after which the medium is exchanged with fresh medium. Aliquots of the bead culture are monitored for rickettsial growth by cytofuging trypsinized cells onto glass slides (cytofuge) which are then stained by the method of Giménez.

Under the conditions described, 85-95% of the cells are infected at approximately two hours post-infection. At 72 hours post-infection there are greater than 50 rickettsiae per infected cell. The cultures are harvested either 72 or 96 hours post-infection by first blending the culture in an Omni-mixer and then by using differential centrifugation to separate the beads and cells from the rickettsiae. The rickettsiae are pelleted and then purified on a Renograffin equilibrium gradient. The microcarriers aid in host cell debris removal as the host cell debris reattaches to the beads and is easily centrifuged away from the released rickettsiae.

b. Preparation of ^{3}H-labeled rickettsiae of a high specific activity. The microcarrier culture system described above is used to produce radioactively labeled rickettsiae. Cycloheximide or emetine is used to inhibit host cell protein synthesis when there are approximately 15 rickettsiae per infected cell. After 24 hours of pre-treatment with cycloheximide (or emetine) the medium is replaced with leucine-free medium and 3% dialyzed fetal calf serum. After 4 hours, during which time leucine pools are depleted, the medium is again replaced and 50 µCi per ml of ^{3}H-leucine is added. The culture is incubated with the radioactive leucine for 24 hours, at which time it is harvested and purified on Renografin gradients. The purified labeled rickettsiae are then used in analytical techniques to be described below. This system yields from 10^{6} to 10^{7} TCA precipitable counts of purified rickettsiæ from one 25 ml bead culture.

Tritium or 35 S labeled proteins separated in polyacrylamide gels were visualized by the fluorographic procedure of Bonner and Laskey (1). The gels were impregnated with PPO and dried down on filter paper. The dried gels were then exposed to Kodak Blue Brand film at -70°C.

Specific rickettsiæ protein profiles on gel electrophoresis were achieved with non-purified rickettsiæ grown in L-cells treated with emetine (1 or 2 µg/ml). Emetine specifically inhibits host cell protein synthesis (95% inhibition). Emetine treated L-cell monolayers infected with R. prowazekii are labeled with ^{3}H-leucine or ^{35}S-methionine for 24 hours and are then harvested by adding hot SDS-electrophoresis sample buffer directly to the monolayer. Both L-cell and rickettsial proteins are solubilized and loaded onto discontinuous slab gels. Coomassie stained protein profiles of emetine treated infected L-cells, emetine treated L-cells and L-cells alone all look the same. However, by fluorography the protein profiles are much different. Emetine inhibits practically all host cell protein synthesis and in infected cells treated with emetine the profile is that of R. prowazekii. This procedure allows rapid monitoring of rickettsial protein synthesis and will greatly enhance our ability to analyze different strains or mutants of rickettsiæ without extensive purification.

2. Analysis of R. prowazekii proteins. The DNA of R. prowazekii with a molecular weight of 1.2×10^{9} daltons can theoretically code for approximately 1400 proteins of average molecular weight (2). To date, the maximum number of polypeptides reported is 64 in the Madrid E strain of epidemic typhus as analyzed by polyacrylamide gel electrophoresis (3). Comparisons of virulent R. prowazekii Breinl strain with the attenuated E strain show no differences on polyacrylamide gels, although a difference was noted by isoelectric focussing (4). Therefore, since only a small fraction of the theoretical number of proteins of R. prowazekii is being resolved by one-dimensional gel electrophoresis and strain differences are not detected, we chose to use the sensitive technique of 2-dimensional electrophoresis as developed by O'Farrell (5) for analysis of intrinsically labeled radioactive proteins of R. prowazekii. This system can resolve 1100 different polypeptides of E. coli (5) and has recently been used to detect differences between virulent and avirulent strains of Mycoplasma pneumoniae (6).

a. Two-dimensional electrophoresis of intrinsically labeled radioactive proteins of R. prowazekii. Rickettsia prowazekii was intrinsically labeled with tritiated leucine and purified on Renografin gradients

as described above. Approximately 500,000 TCA precipitable counts of labeled rickettsiæ were digested in a lysis buffer which consisted of 2% Triton X-100, 8 M urea, 2% ampholytes (pH 3-10) and 5% beta-mercaptoethanol. The digestion was carried out at 4°C to prevent multiple-spot formation in the gels due to carbamylation of amino groups by urea (7).

The first dimension electrophoresis was performed on a flatbed analytical isoelectric focussing gel. A flatbed gel was used since (1) it allows many samples to be applied and focussed under identical conditions; (2) the pH gradient can be easily measured with a surface electrode and (3) the samples can be rapidly sliced out of the gel and placed on a SDS-polyacrylamide slab gel for the second dimension. The first dimension gels consisted of 5% acrylamide cross-linked with BIS-acrylamide, 2% Triton X-100, 8 M urea and 2.5% ampholytes (pH range 3-10). Prefocussed gels were loaded at the anode end with a small rectangular piece of Whatman filter paper saturated with the digested sample. 25 watts constant power was applied for 3 hours at 6°C after which pH readings were taken at 1 cm intervals with an inc ld surface electrode. The gel was then reequilibrated for 30 minutes. Gel slices containing individual samples were then cut according to a template placed under the gel. Each slice is immersed in 5 mls of SDS-electrophoresis sample buffer in preparation for the second dimension. The SDS sample buffer contains 0.005 M phosphate buffer, 5% beta-mercaptoethanol, 3% SDS, 10% glycerol and 0.005% bromphenol blue.

The second dimension gel is a discontinuous gradient gel modified from O'Farrell (5). The gradient gel is 9-18% acrylamide cross-linked with DATD (N, N'-dialytartardiamide), which is overlayed with a 4 cm high, 5% acrylamide stacking gel. Special notched plates are used which allow the first dimension gel to lie on top of the SDS-slab gel. Constant concentration acrylamide gels were used in some experiments for the second dimension but it was found that the small molecular weight proteins did not resolve well. The gradient gels are designed to resolve both large and small molecular weight proteins. The second dimension is electrophoresed at constant voltage (90 volts) for 15 hours. Standard preparations of labeled R. prowazekii were run only in the second-dimension SDS slab gel to be used as a marker.

Two dimensional electrophoresis of tritiated proteins of R. prowazekii (Breinl) reveal over 150 different polypeptide species. This represents at least a 3-fold increase in the number of polypeptides reported to date for R. prowazekii. Preliminary results show that when the virulent (Breinl) train is compared to the attenuated (Madrid E) strain by 2-dimensional electrophoresis, unique polypeptides can be found for each strain (see Figures 19 and 20); however, the majority of proteins are superimposable when the gels are placed on top of each other. Thus, separating radioactive proteins first by their isoelectric points and then by molecular weight greatly increases the sensitivity for detecting genetic differences in rickettsial organisms.

b. Identification of specific protein antigens of R. prowazekii. The rickettsiae are complex organisms which contain many potential immunogens. The slime layer is undefined biochemically but probably consists of carbohydrate that may be immunologically recognized. In addition R. prowazekii also contains at least one surface protein (8) that may function in adsorption to, and penetration of, host cell membranes. Surface proteins are also potential immunogens and antibody to them may be an important host defense.

FIGURE 19

Two-dimensional Electrophoretic Analysis of ^{3}H Leucine Labeled Proteins from R. prowazekii (Breinl).

Over 150 different polypeptides can be identified on the original fluorograph at one week's exposure time. The arrows indicate polypeptides unique to the Breinl strain when compared to the Madrid E strain.

Uniqueness is determined by superimposing 2-D gel fluorographs of E and Breinl that were run in parallel in both the first and second dimension.

FIGURE 19

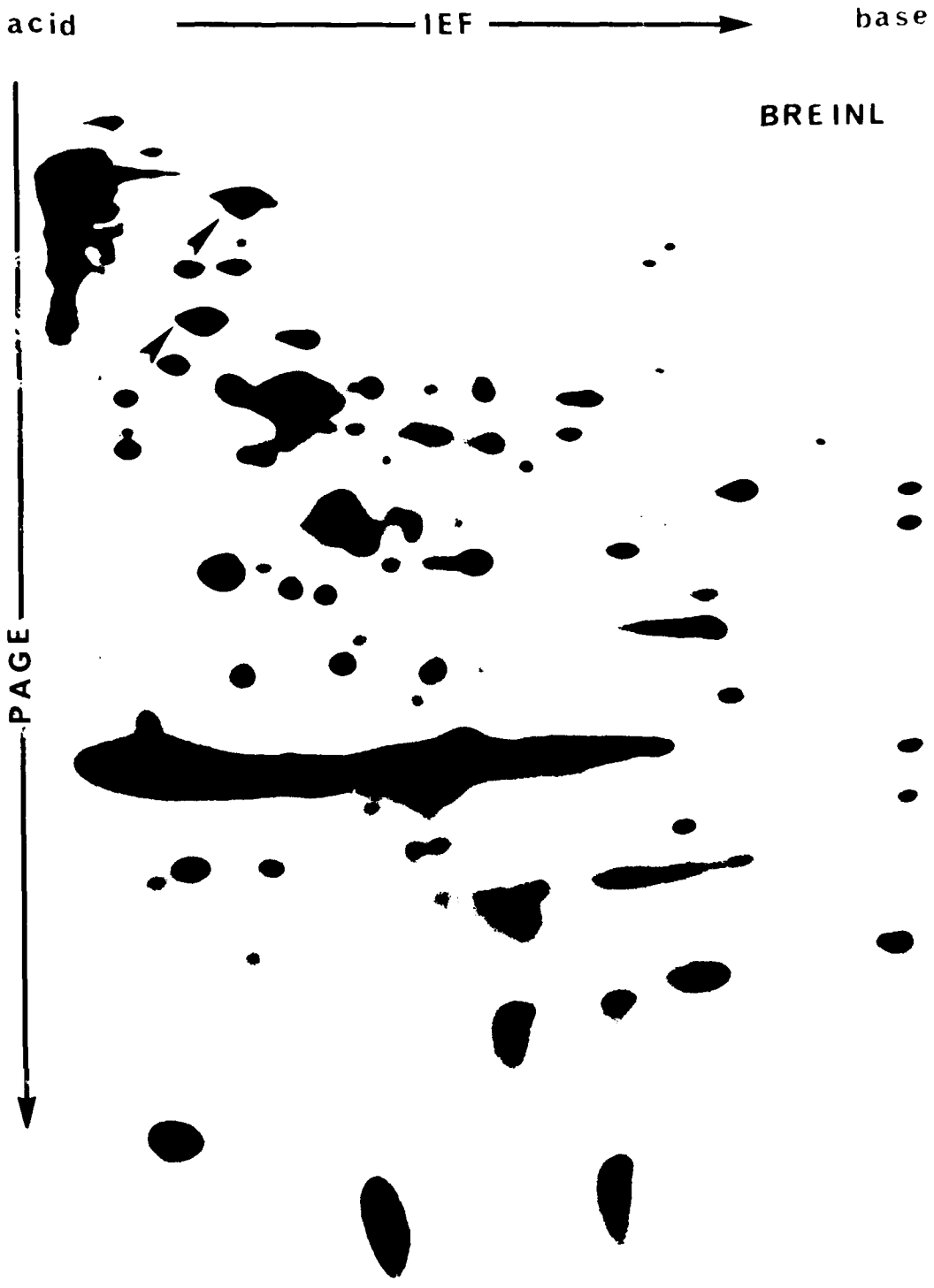
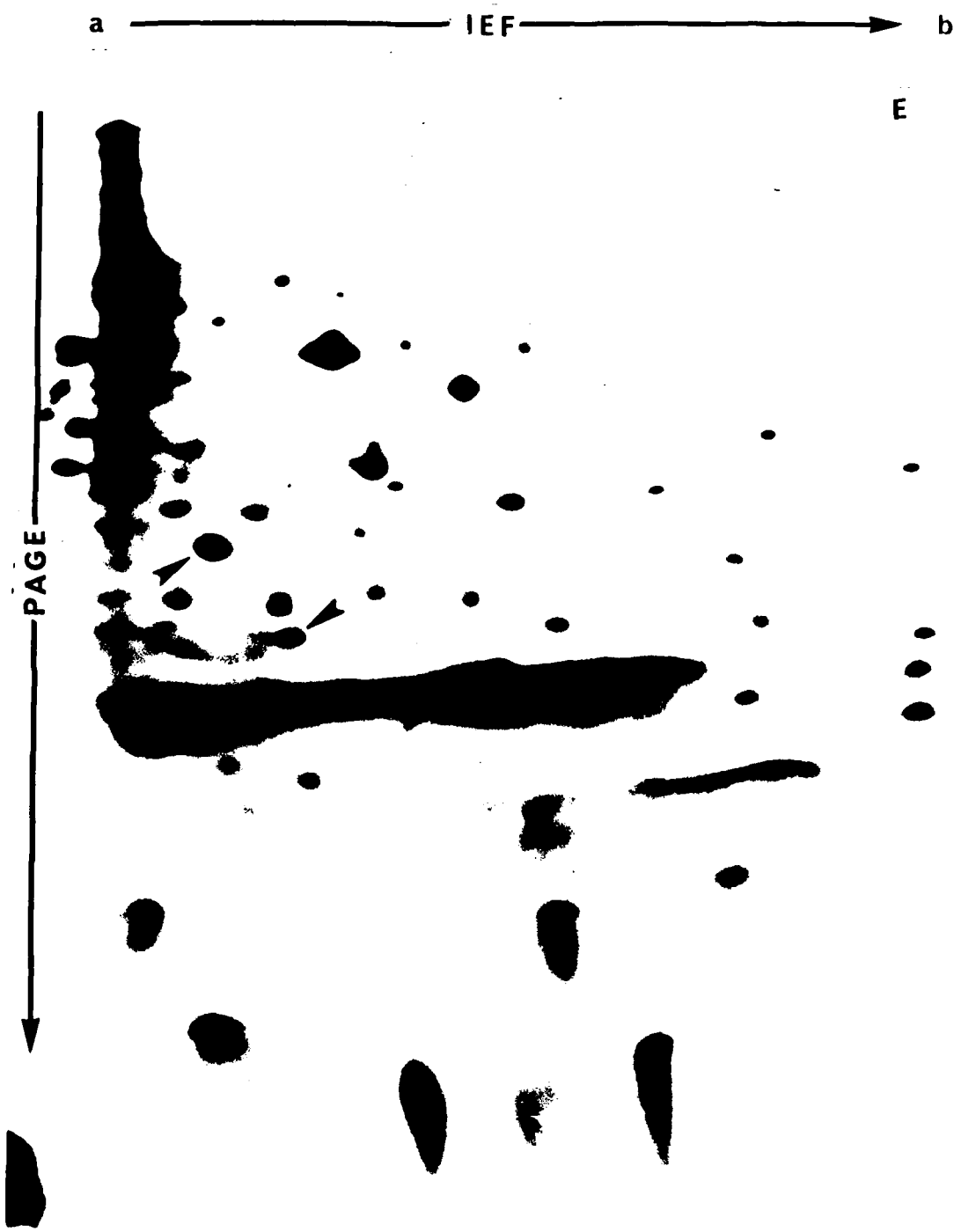


FIGURE 20

Two-dimensional Electrophoretic Analysis of ^{3}H Leucine Labeled Proteins from R. prowazekii (Madrid E).

Polypeptides unique to the Madrid E strain of R. prowazekii are indicated by arrows. The large smear in the upper left hand corner is due to incomplete solubilization of rickettsial proteins which consequently are unable to migrate in the isoelectric focussing dimension.

FIGURE 20



To identify rickettsial protein antigens we used a system to solubilize rickettsial proteins under non-denaturing conditions which allows precipitation with immune serum.

(1) Protein antigens of renograffin purified R. prowazekii. ^{3}H-leucine labeled R. prowazekii purified on Renograffin gradients was solubilized in 2% Triton X-100 and 6 M urea at 4°C. After solubilization the digest was dialyzed against 2% Triton X-100 in phosphate buffered saline to remove the urea. The digest was recovered and centrifuged at 20,000 xg for 30 minutes to remove any insoluble matter. Next the supernatant was reacted with immune human serum (convalescent) for 12-16 hours at 4°C. To this mixture was added 25 mg of Staphylococcus aureus cells containing protein A on its surface (Pansorbin, Calbiochem) which will bind IgG molecules by the F_c end. Antigen bound to IgG molecules will thus be indirectly linked to the Staph protein A which can be easily separated from unreacted labeled proteins by centrifugation. The Staph protein A pellet is washed three times and then treated with SDS-electrophoresis sample buffer which solubilizes antigen-antibody complexes coupled to the pellet. The sample is then boiled for two minutes, centrifuged and the supernatant is loaded onto SDS-polyacrylamide gels. Controls for non-specific reactions include non-immune serum as well as PBS in the place of serum.

^{3}H-leucine labeled proteins of renograffin purified rickettsia were solubilized by Triton X-100 and urea and subsequently incubated with immune human sera, normal human sera or PBS. To these mixtures 50 mg of Staph protein A (Calbiochem) was added to adsorb IgG and IgG-antigen complexes. The adsorbed complexes were centrifuged down, washed three times, and finally solubilized in SDS-electrophoresis sample buffer. Aliquots of these samples were electrophoresed on discontinuous SDS-polyacrylamide gels.

Both the Breinl and attenuated E strains of R. prowazekii show a high molecular weight and a low molecular weight protein that is precipitated only by immune serum (human convalescent).

(2) Identification of protein antigens of non-purified R. prowazekii. The previous section described a procedure used to solubilize and identify protein antigens of purified rickettsiae. However, it is possible that some components may have been lost or altered during purification. It is also possible that all proteins are not solubilized. Therefore, we developed a system where protein antigens of non-purified rickettsiae can be solubilized and identified by immune selection.

Non-ionic detergents (Triton X-100) do not have the capacity to solubilize all rickettsial proteins from intact cells (unpublished observations) but they do not inhibit antigen-antibody reactions (9). On the other hand detergents with very high solubilizing capacity, such as the anionic detergent SDS, will completely inhibit antigen-antibody reactions by protein denaturation (9). To optimize solubilization and also allow immune selection we chose to solubilize with SDS and then replace the SDS with the non-ionic detergent, Triton X-100, which allows antibody-antigen reactions to occur.

R. prowazekii (Breinl) was grown in L-cell monolayers. L-cell protein synthesis was inhibited with 1 µg/ml emetine 24 hours prior to labelling with either ^{3}H-leucine or ^{35}S-methionine. After the 24 hour pre-treatment with emetine, the culture medium was replaced with either leucine-free or methionine-free medium containing 3% dialyzed fetal calf serum and 1 µg/ml emetine. To this was added the radioactive amino acid, ^{3}H-leucine (50 µCi/ml) or ^{35}S-methionine (10 µCi/ml). Incorporation of the isotope was allowed to occur for 24 hours at which time the monolayers were scraped into the medium, centrifuged at 20,000 xg for 30 minutes and then solubilized in 2% SDS. Alternatively, SDS electrophoresis sample buffer could be added at this step in preparation for polyacrylamide gels, if immune selection procedures are not to be used. The sample solubilized in 2% SDS is then run through a hydroxylapatite column equilibrated with 0.001 M phosphate buffer, pH 6.8. The column is then washed with 2% Triton X-100 in 0.001 M phosphate until counts in the eluates reach background levels. At this time the buffer is changed to 2% Triton X-100 in 0.5 M phosphate. This buffer allows all proteins to be eluted. Aliquots of all fractions containing TCA counts are then immune precipitated with immune human sera and staph protein A. The precipitates are monitored by SDS-polyacrylamide gel electrophoresis.

Emetine treated L-cells containing approximately 100 rickettsiae per cell were solubilized with 2% SDS after 24 hours of growth in the presence of ^{35}S-methionine. The digest was applied to a hydroxylapatite column which was then washed with a low phosphate (0.001 M) buffer containing 2% Triton X-100 and then a high phosphate (0.5 M) buffer containing 2% Triton X-100. Aliquots of fractions containing peak radioactivity were treated with immune human serum or normal human serum and subsequently adsorbed to Staph protein A. The staph protein A pellets were digested with SDS-electrophoresis sample buffer and then applied to SDS-polyacrylamide gels.

Figure 21, columns 5, 6 and 7, show that the same high molecular weight protein immunoprecipitated from purified rickettsiae is also immunoprecipitated from purified rickettsiae is also immunoprecipitated from nonpurified rickettsiae solubilized by SDS. Many other proteins are also adsorbed out by the Staph protein A but are in the normal serum treated samples and are therefore considered background adsorption. The use of the hydroxylapatite column allows exchange of detergents thereby permitting the use of antibody for immune selection of rickettsial proteins.

3. References

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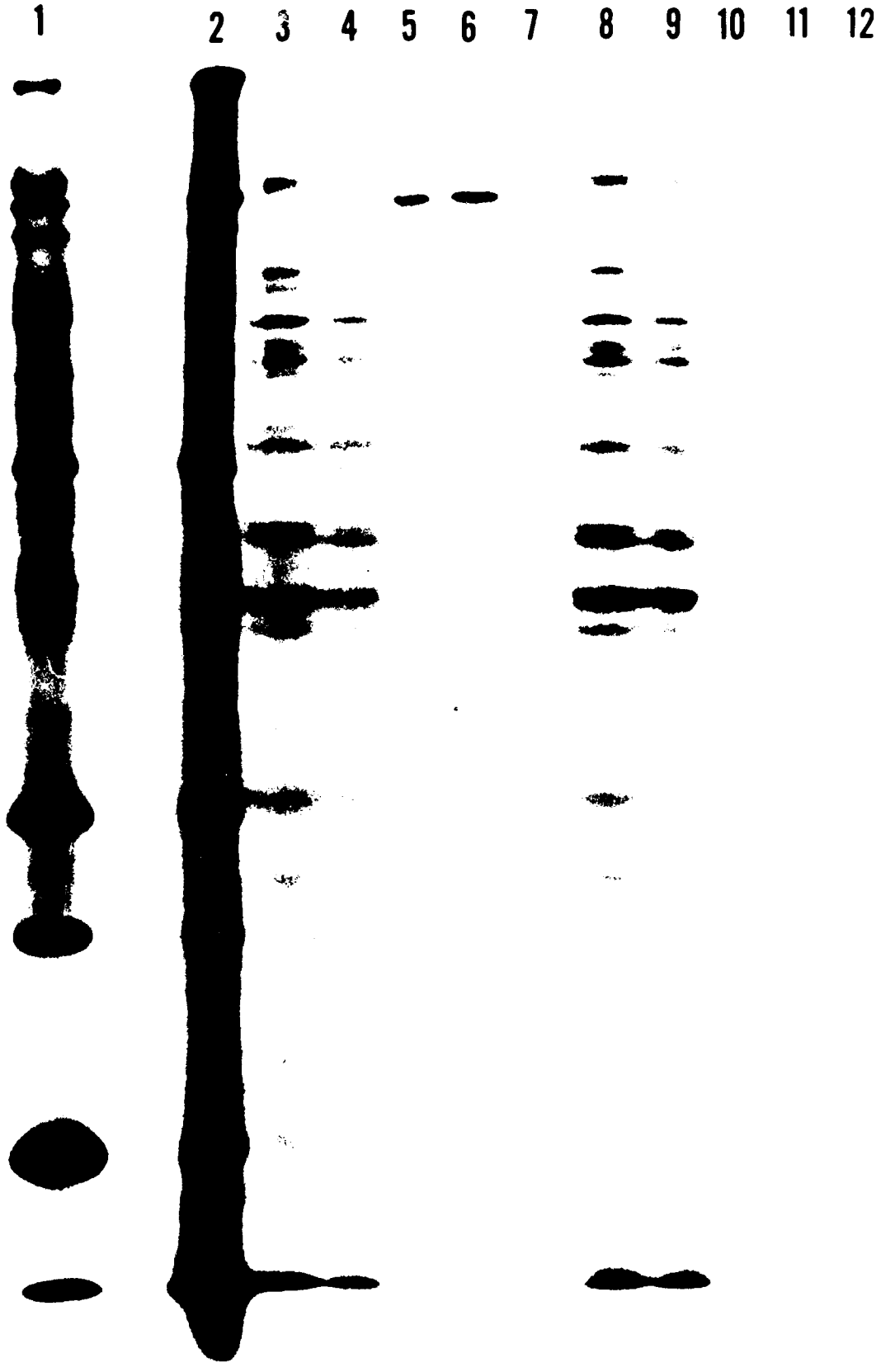
FIGURE 21

Immune Selection of SDS-Solubilized Proteins of Non-Purified R. prowazekii

SDS-solubilized R. prowazekii proteins (^{35}-S-methionine labeled in emetine treated L-cells) eluted off a hydroxylapatite column in the presence of Triton X-100 were treated with either immune human serum (columns 3-7) or non-immune human serum (columns 8-12). Columns 5, 6 and 7 reveal a high molecular weight protein precipitated only by immune human serum. Columns 3 and 4 contain many proteins of rickettsial origin but are considered non-specific precipitates since they are also present in columns 8 and 9 which were treated with non-immune serum. Column 1 shows the rickettsial proteins that are not solubilized by SDS treatment alone and column 2 is an over-exposed pattern of ^{35}-S-labeled R. prowazekii in the presence of emetine treated L-cells.

FIGURE 21



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C. Proteins of R. tsutsugamushi (Hanson, Oaks, Smith)

The causative organisms of scrub typhus have in common certain biological properties and also share antigens to varying degrees. Nevertheless, these rickettsiae can be divided into several strains on the basis of serological identification and biological characteristics such as virulence. The patterns of serological cross-reactivity are complex, defying a simple classification of scrub typhus strains into subgroups by antigenic typing. The occurrence of strain variation presents a medical problem in terms of susceptibility to reinfection and development of an effective vaccine. On the other hand, it provides a tool for studying rickettsia-host interactions in a scientific area which suffers from a lack of mutant organisms with which to experiment.

Because R. tsutsugamushi is difficult to grow in quantity and to purify from host cell contaminants, it has not been studied to the same extent that other rickettsiae have been studied by modern methods. However, the methods described in "B" above, which permit selective radiolabeling of rickettsial proteins and the demonstration of rickettsial protein PAGE profiles from unpurified, radiolabeled tissue culture grown organisms through the application of radiofluorography, gave hope that, for the first time, protein profiles of different serotypes of R. tsutsugamushi could be prepared.

The initial step has been an analysis of the proteins of three major antigenic varieties of R. tsutsugamushi: the Gilliam and Kato strains, both with long histories of egg passage, and JC472B, a relatively recent isolate which resembles the Karp strain antigenically (1). The organisms were grown in tissue cultures in the presence of ^{3}H-leucine and emetine, an antibiotic which specifically inhibits eukaryotic protein synthesis. The labeling protocol was developed in this laboratory by Mr. Ed Oaks. The proteins of whole cell extracts subsequently were analyzed by polyacrylamide gel electrophoresis and fluorography.

As shown in the accompanying figure (Figure 22), the emetine treatment of uninfected cells shut down most of the background labeling of host proteins. Careful comparison with infected cells indicates that rickettsial infection further contributed to the inhibition of host cell protein synthesis. Emetine-resistant protein synthesis in infected cells, presumed to be rickettsial, was largely inhibited by chloramphenicol, which specifically inhibits bacterial, but not eukaryotic, protein synthesis.

The protein profiles of the three strains of R. tsutsugamushi examined show some remarkable differences in both minor and major proteins, the latter of which may be structural. Other major proteins appear to be shared by all three of the rickettsial variants.

Studies are underway to determine how these proteins relate to the rickettsiae's antigenic properties and the biological significance of some of the proteins. The latter will involve analysis of scrub typhus isolates obtained in Pakistan, which are serologically more closely related than the three prototypes analyzed so far, but which differ greatly in their virulence and perhaps other functional properties (1).

The immunoprecipitation of radiolabeled rickettsial proteins followed by polyacrylamide gel electrophoresis and radiofluorography, as described in "C" above may yield precise information on the shared and unique protein antigens of different strains and serotypes and may eventually permit description of strains in terms of precise antigenic formulae.

In addition to providing a basis for understanding the antigenic structure and function of scrub typhus organisms, the protein profiling of isolates from different hosts and geographic regions may lead to a clearer picture of the evolution and dissemination of this disease.

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D. Initial interaction between R. prowazekii and chicken embryo cells (CEC) (Hanson)

We are investigating the nature of the initial interaction of R. prowazekii with cultured chick embryo cells (CEC), with an emphasis on the surface molecules required for attachment and penetration.

1. Technical. A method to assay attachment/penetration was developed, using purified rickettsiae labeled by incubation with ^{3}H-ATP, as first described by Walker and Winkler (1). Freshly labeled rickettsiae were mixed with CEC in suspension; rickettsial association with host cells was determined by centrifugation of the CEC and analysis of the radioactivity in the supernate which was still rickettsia-associated (i.e., was retained on 0.45 μm pore size Millipore filters). This technique, although not as precise as microscopically counting cell-associated rickettsiae, was rapid and entirely adequate for the comparative experiments planned. The

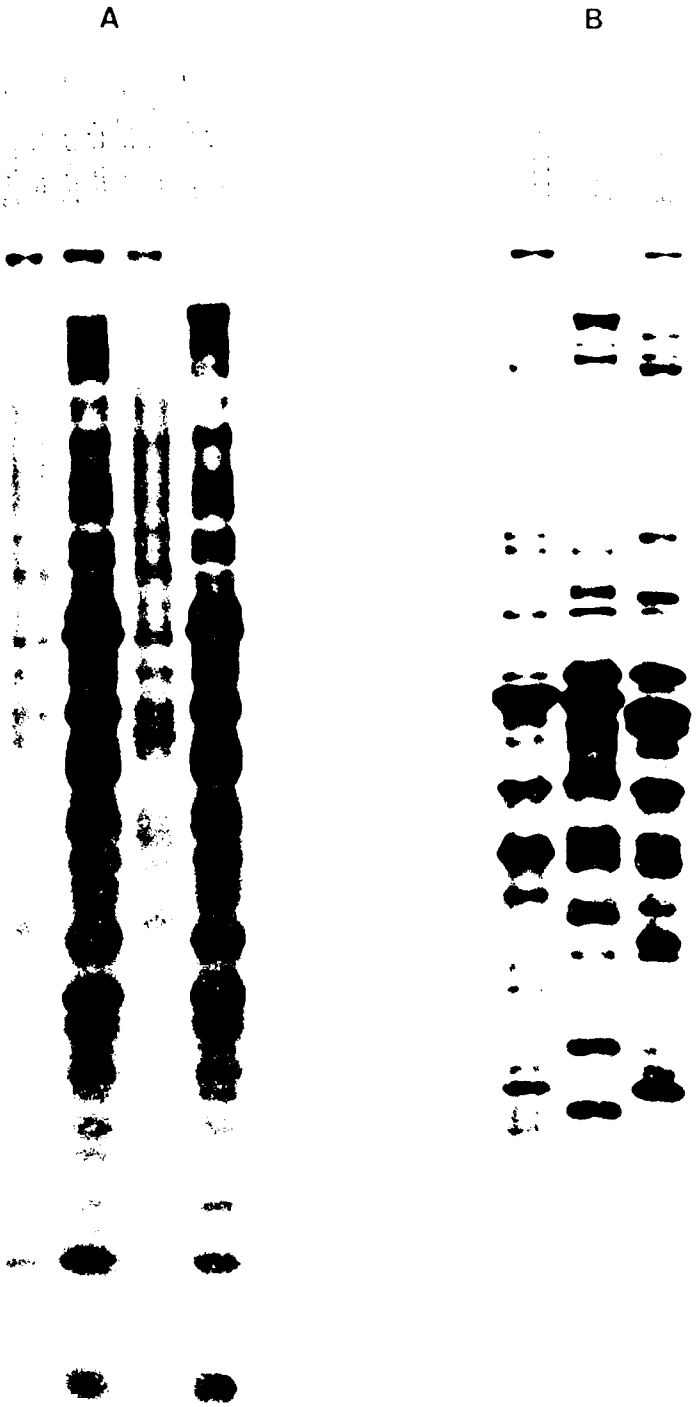
FIGURE 22

Single-dimensional Polyacrylamide Gel Electrophoresis Patterns of 3 R. tsutsugamushi Serotypes.

Panel A is a fluorograph of PAGE profiles of emetine-inhibited, radiolabeled uninfected L cells and L cells infected with the JC 472B strain of R. tsutsugamushi (a Karp-like strain from Pakistan). In other experiments, it was shown that the infection itself further reduced host-cell labeling and that putative rickettsial proteins were suppressed in the presence of chloramphenicol.

Panel B is a fluorograph of PAGE protein profiles of 3 serotypes of R. tsutsugamushi: Gilliam, JC 472B (Karp-like) and Kato.

FIGURE 22



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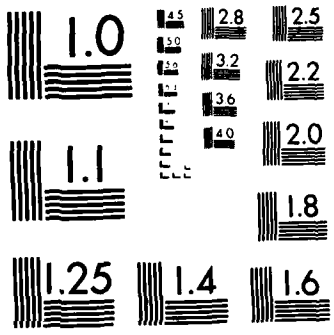

kinetics and temperature dependence of rickettsial attachmen/penetration in this system are similar to that observed by direct visual counting.

2. Effect of rickettsial treatments on their association with CEC. Penetration of cells by rickettsiae requires the active participation of both microorganism and host. A surface structure on the rickettsiae might act as a receptor to specifically bind to target cell membranes, or as a component which actively alters the host cell membrane (e.g., a toxin or enzyme). In an attempt to identify such structural components, rickettsiae were pre-treated with a number of reagents which might alter their external surface. Exposure of the organisms to Tween 80, neuraminidase, periodate, trypsin, pronase, phospholipase A or phospholipase C had no effect on their subsequent ability to attach to CEC. Neither did any of these treatments affect the rate at which ^{3}H-ATP was released from labeled rickettsiae in the absence of CEC.

3. Interaction of rickettsiae with cholesterol. Cholesterol in sheep erythrocyte membranes is responsible for at least part of their rickettsial receptor activity (2). Experiments were done to explore the nature of the interaction between rickettsiae and cholesterol and to determine if cholesterol might be similarly involved in the attachment of R. prowazekii to chick cells.

a. Binding of ^{3}H-cholesterol to rickettsiae. ^{3}H-cholesterol bound to rickettsiae rapidly; Tween 80, added to solubilize the steroid, slowed the binding considerably, and enabled kinetic studies which showed the temperature dependence of the binding. The binding of cholesterol was probably a physical, absorptive process, as has been shown with other microorganisms: Heat-killed rickettsiae were only slightly inhibited in their ability to bind cholesterol; bound cholesterol eluted from the organisms only when the equilibrium was upset by the removal of cholesterol from the suspending medium. The ^{3}H-cholesterol appeared to be bound by rickettsial membrane protein because 1) cholesterol binding was prevented by pre-treatment of rickettsiae with trypsin or pronase and 2) ^{3}H-cholesterol bound to rickettsiae was precipitable by cold TCA.

b. Role of cholesterol in the association of rickettsiae with CEC. Pre-treatment of CEC with digitonin, a polyene antibiotic whose target is cholesterol, dramatically inhibited attachment/penetration of rickettsiae with the subsequently washed cells. This indicated either that cholesterol acts as part of the target cell receptor complex or that it is required indirectly to maintain a proper structural or functional organization in the host membrane. To distinguish between these, the steroid was tested for activity as a competitive inhibitor. Rickettsiae were preincubated with cholesterol and then were added to CEC, still in the presence of cholesterol. This treatment had no effect on the association of the microorganisms with the target cells, suggesting that cholesterol is not part of the host receptor per se.

Further evidence that rickettsiae are not required to bind to a cholesterol-containing receptor as part of the infectious process comes from the following comparison. Pre-treatment of R. prowazekii with Tween 80,

trypsin, or pronase profoundly inhibited their binding to cholesterol but none of these treatments affected the association of the microorganisms with the target cells.

4. Conclusion. The preliminary tests with a number of enzymes have failed to identify the chemical nature of a rickettsial membrane site required for attachment to or penetration of host cells. On the other hand, we have eliminated the possibility of a direct role for cholesterol as part of a target cell receptor site, although cholesterol may be important in maintaining the membrane in a "receptive state". In addition, the demonstration of the ability of rickettsiae to readily bind cholesterol leads to intriguing questions about the effect this property might have on the stability of the organisms in their natural invertebrate and mammalian hosts.

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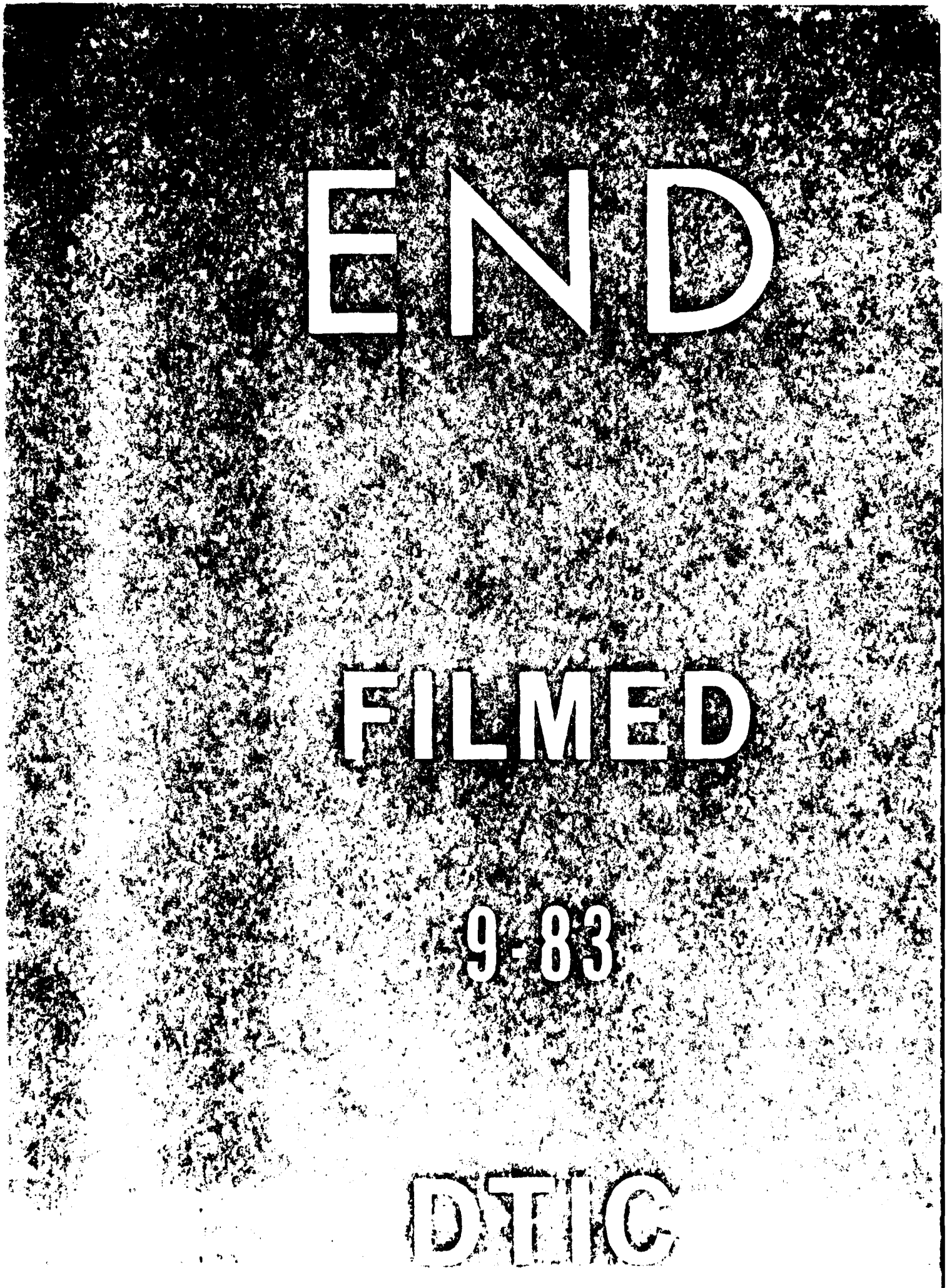
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